Chimeric L-type Ca^{2+} channels expressed in *Xenopus laevis* oocytes reveal role of repeats III and IV in activation gating

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1. Chimeric α_1 subunits consisting of repeat I and II from the rabbit cardiac ($\alpha_{1C\text{-}a}$) and repeat III and IV from the carp skeletal muscle Ca^{2+} channel (α_{1S}) were constructed and expressed in *Xenopus laevis* oocytes without co-expressing other channel subunits. Ba^{2+}-current kinetics of five chimeric channel constructs were studied in *Xenopus* oocytes using the two-microelectrode technique.

2. Exchange of repeats III and IV of $\alpha_{1C\text{-}a}$ with sequences of α_{1S} results in a significantly slower and biexponential activation (apparent activation time constants $\tau_{1act} = 19{\cdot}8 \pm 1{\cdot}8$ ms and $\tau_{2act} = 214 \pm 28{\cdot}7$ ms, $n = 7$) of expressed Ca^{2+} channel currents; no current inactivation was observable during an 800 ms test pulse to 0 mV.

3. Activation of a chimera consisting of repeats I, II and IV from the $\alpha_{1C\text{-}a}$ subunit and repeat III from α_{1S} was fast and monoexponential ($\tau_{1act} = 6{\cdot}33 \pm 1{\cdot}7$ ms, $n = 5$) and the current inactivated during a 350 ms test pulse to 0 mV ($\tau_{inact} = 175 \pm 22$ ms, $n = 5$). The current kinetics of this construct did not significantly differ from kinetics of a construct consisting of repeats I to IV from $\alpha_{1C\text{-}a}$ ($\tau_{1act} = 6{\cdot}6 \pm 2{\cdot}1$ ms; $\tau_{inact} = 198 \pm 14$ ms; $n = 9$).

4. Expression of a chimera where only the region IIIS6 to IVS5 and adjacent amino acids were changed from the cardiac $\alpha_{1C\text{-}a}$ to α_{1S} yielded channels that activated slowly at 0 mV with a biexponential time course (apparent $\tau_{1act} = 12{\cdot}9 \pm 1{\cdot}9$ ms and $\tau_{2act} = 262 \pm 18{\cdot}9$ ms, $n = 6$).

5. Single channel conductances of a fast ($19{\cdot}4 \pm 1{\cdot}1$ pS) and a slow activating chimera ($18{\cdot}6 \pm 1{\cdot}2$ pS) were estimated with 90 mM Ba^{2+} as charge carrier to be close to the conductance of L-type Ca^{2+} channels.

6. These results demonstrate that repeats III and IV of α_{1S} from carp skeletal muscle possess structural determinants for 'slow activation' of L-type Ca^{2+} channels.

Different types of voltage-dependent Ca^{2+} channels (T-, L-, N- and P-type) are distinguished according to pharmacological, biophysical and structural criteria (Tsien, Ellinor & Horne, 1991; Hofmann, Biel & Flockerzi, 1994; Birnbaumer *et al.* 1994). L-type Ca^{2+} channels represent a structurally heterogeneous group of cardiac, smooth muscle, neuronal, neuroendocrine and skeletal muscle channel proteins encoded by different genes that share common pharmacological properties (Striessnig, Berger & Glossmann, 1993; Hofmann *et al.* 1994; Birnbaumer *et al.* 1994). The pore-forming α_1 subunits of various L-type channels form hetero-oligomeric structures with β, α_2-δ and γ subunits modulating channel gating, density of expressed currents and drug-binding properties (for review see Hofmann *et al.* 1994).

Despite common pharmacological features, including sensitivity to 1,4-dihydropyridines, subtypes of L-type Ca^{2+} channels exist with respect to their functional properties. Thus, α_{1S} channels activate with a significantly slower time course than other L-type Ca^{2+} channels (Sanchez & Stefani, 1983; Beam, Adams, Niidome, Numa & Tanabe, 1992; Caffrey, 1994). Because of their localization in transverse tubular membranes of skeletal muscle fibres, a precise biophysical characterization of α_{1S} current kinetics was complicated in native muscle fibres. Progress was achieved by cloning of the rabbit skeletal muscle α_{1S} subunit (Tanabe *et al.* 1987) and the subsequent expression in L-cells (for review see Hofmann *et al.* 1994). The identification of the α_{1S} subunit in a myogenic cell line

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(BC3H1; Biel *et al.* 1991) enabled a detailed characterization of this channel type in single cells (Caffrey, 1994). One of the most interesting questions resulting from the molecular and functional diversity of Ca^{2+} channels is the identification of structural elements which determine functional properties. Two structural motifs that influence Ca^{2+} current kinetics have been identified recently in repeat I of α_1 subunits (Tanabe *et al.* 1991; Nakai *et al.* 1994; Zhang, Ellinor, Aldrich & Tsien, 1994).

The aim of this paper was to determine if repeats III and IV of L-type α_1 subunits have relevance for functional channel properties. We made use of the different kinetic properties of heterologously expressed chimeras of $\alpha_{1C\text{-}a}$ (Mikami *et al.* 1989; Sather, Tanabe, Zhang, Mori, Adams & Tsien, 1993) and α_{1S} Ca^{2+} channels (in this study an α_{1S} subunit from carp skeletal muscle; see Grabner *et al.* 1991). We demonstrate that regions other than those recently identified in repeat I (Tanabe *et al.* 1991; Nakai *et al.* 1994), namely motifs of repeats III and IV, are able to modulate activation gating of L-type Ca^{2+} channels. Single channel studies revealed that implantation of α_{1S} repeat IV in $\alpha_{1C\text{-}a}$ does not significantly affect the conductance of the channel pore.

Preliminary findings have been communicated (Wang, Savchenko, Grabner, Froschmayr, Hering & Glossmann, 1995).

METHODS

Construction of chimeric α_1 cDNAs

Chimeric constructs were ligated into polylinker sites of the polyadenylating pBluescript derivative pNKS2 (generously provided by Professor O. Pongs). Chimeras between rabbit heart $\alpha_{1C\text{-}a}$ (H) (Mikami *et al.* 1989) and carp skeletal muscle α_{1S} (S) (Grabner *et al.* 1991) were constructed as follows.

sH. (Amino acid numbers in parentheses) S(1–60), H(145–2171). The KpnI–AccIII fragment (nucleotide numbers in parentheses) of H (polylinker–620) was replaced by a KpnI–AccIII fragment of S (polylinker–360) using a common AccIII restriction site of a KpnI–BamHI (polylinker–1456) H-subclone in pBluescript SK^+. This shortening of the 5′-region yielded higher expression levels in yeast (unpublished data) compared with unmodified $\alpha_{1C\text{-}a}$ cDNA and was therefore routinely used in our expression studies. Replacing the amino terminus by this sequence from α_{1S} did not influence the Ca^{2+} channel current kinetics measured after expression of $\alpha_{1C\text{-}a}$ in *Xenopus* oocytes (results not shown) but resulted in a significantly higher current density observed as an increase in peak Ba^{2+} current (I_{Ba}) from $I_{Ba,H} = 197{\cdot}8 \pm 17{\cdot}4$ nA ($n = 12$) to $I_{Ba,sH} = 423 \pm 51{\cdot}4$ ($n = 17$) (measured at 20 mV test potential in three batches of oocytes). As kinetics of $\alpha_{1C\text{-}a}$ currents were not affected, all chimeras were designed by replacing part of the $\alpha_{1C\text{-}a}$ amino terminus by this α_{1S} sequence (S(1–60)).

To construct the following H/S α_1-chimeras (as depicted in Fig. 1*A*), PCR primers were used to create three specific restriction sites by introducing mutations converting H to S or S to H (mutational sites are indicated by an asterisk).

sHHSSs. S(1–60), H(145–920), S(806–1852). This half–half chimera was assembled by ligating the BamHI–HindIII* fragment of H(1456–2953) together with a HindIII–XhoI fragment of S(2600–polylinker) into the BamHI(1456H) and XhoI(6923H) sites of sH.

sHHSSh. S(1–60), H(145–920), S(806–1347), H(1450–2171). The BamHI–HindIII* fragment of H(1456–2953) was ligated together with the HindIII–EcoRV* fragment of S(2600–4228) into the BamHI(1456H) and EcoRV(4542H) sites of sH.

sHHHSh. S(1–60), H(145–1159), S(1045–1347), H(1450–2171). Fragment BamHI–HindIII* of H(1456–2953) was co-ligated with the HindIII*–SalI* fragment of H(2953–3670) into the BamHI(1456H) and SalI(3317S) sites of SHHSSh.

sHHSHh. S(1–60), H(145–920), S(806–1046), H(1162–2171). The SalI*–EcoRV fragment of H(3670–4542) was inserted between the SalI (3317S) and EcoRV (4542H) sites of SHHSSh.

Capped run-off poly(A)$^+$ cRNA transcripts from XbaI linearized cDNA templates were synthesized and injected into *Xenopus laevis* oocytes as described elsewhere (Grabner *et al.* 1994).

Expression of chimeric α_1 subunits in *Xenopus laevis* oocytes

The experimental set-up for the two-microelectrode voltage clamp of *Xenopus* oocytes and procedures for oocyte preparation have been described previously (Grabner *et al.* 1994). Female *Xenopus laevis* frogs (NASCO, USA) were anaesthetized by exposing them for 15 min to a 0·2% MS-222 (methane sulphonate salt of 3-aminobenzoic acid ethyl ester; Sandoz, Basel, Switzerland) solution before surgically removing parts of the ovaries. Follicle membranes from isolated oocytes were enzymatically digested with 2 mg ml^{-1} collagenase (Type IA, Sigma). Calcium channel currents were studied 2–3 days after cRNA-injection with 40 mM Ba^{2+} as a charge carrier in a bath solution containing (mM): 40 $Ba(OH)_2$, 40 *N*-methyl-D-glucamine, 10 Hepes, 10 glucose (pH adjusted to 7·4 with methanesulphonic acid). Endogenous chloride currents of the oocytes were suppressed by injecting 20–40 nl of a 0·1 M BAPTA (free acid) solution immediately before the voltage clamp measurements. Voltage steps were applied every 15 s. Leak and capacitative currents were subtracted on-line by scaling the mean leak current of *P*/6 pulses. The I_{Ba} amplitudes of the studied chimeras at 20 mV test potential were usually at least twenty times larger than currents of non-injected oocytes and ranged from several hundred nanoamperes up to several microamperes. All studied chimeric Ca^{2+} channel currents were increased by 1 μM (−)Bay K 8644 (see Table 1). For kinetic studies we selected oocytes with maximum I_{Ba} amplitudes ranging from 200 nA to less than 1 μA to optimize the conditions for voltage clamp. I_{Ba} at 20 mV test potential in non-injected oocytes were either not detectable or ranged from less than 10 to maximal 30 nA (mean peak current, $18 \pm 2{\cdot}1$ nA; $n = 50$; six batches of oocytes) and were insensitive to 10 μM (−)Bay K 8644. For patch clamp experiments, the vitelline membrane of the oocytes was mechanically removed after exposure of the oocytes to a hypertonic solution containing (mM): 200 potassium aspartate, 20 KCl, 1 $MgCl_2$, 5 EGTA, 10 Hepes (pH adjusted to 7·4 with KOH). Single channel currents were recorded by means of an EPC-7 amplifier (List Electronics, Darmstadt, Germany) using the patch clamp technique in the cell-attached configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Patch pipettes were filled with a solution containing (mM): 90 $BaCl_2$, 10 Hepes, 10 glucose

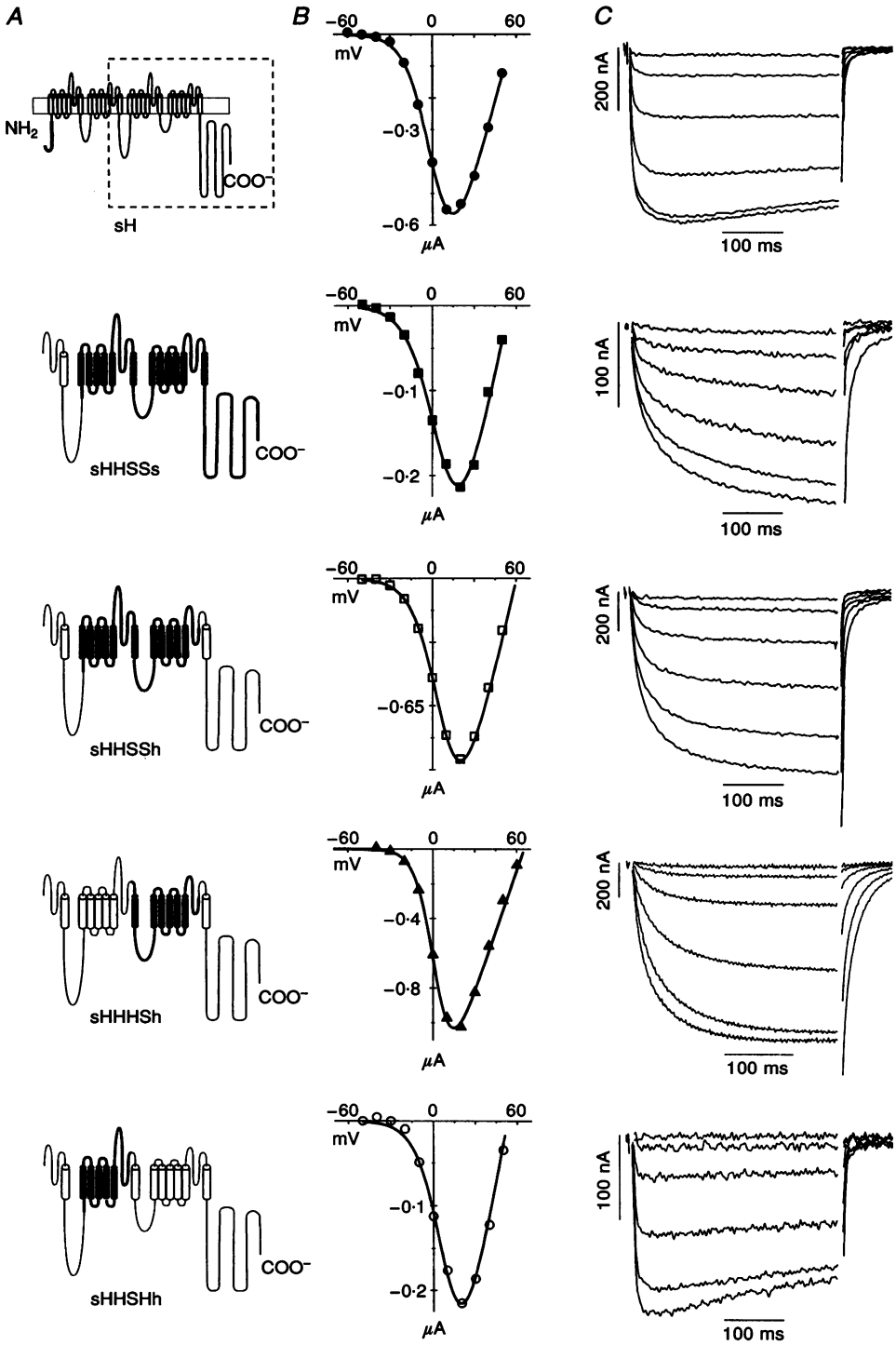

Figure 1. Schematic representation of chimeric α_1 subunits, their current–voltage relationships with corresponding families of Ba^{2+} currents expressed in *Xenopus laevis* oocytes

A, α_1 construct sH composed of repeats I to IV of the cardiac $\alpha_{1C\text{-}a}$ and its chimeras with repeats III and/or IV of the carp skeletal muscle α_{1S} (black cylinders and thick lines) yielding chimeras sHHSSs, sHHSSh, sHHHSh and sHHSHh (see Methods for amino acid composition). As repeats I and II are identical to chimera sH only repeats III and IV are completely shown (boxed in top scheme). *B*, current–voltage relationships of chimeric α_1 subunits. Smooth lines represent the best-fit curves to the function $I_{Ba} = G_{max}(V - V_{rev})/(1 + \exp[(V - V_{0\cdot5})/k])$. I_{Ba}, value of the peak current at a given potential V; G_{max}, maximal conductance value; V_{rev}, reversal potential; $V_{0\cdot5}$, voltage of half-maximal current activation; k, slope factor. The estimated k and $V_{0\cdot5}$ values, respectively, are: sH, −9·04 and 13·6 mV; sHHSSs, −11·4 and 14·82 mV; sHHSSh, −9·27 and 8·75 mV; sHHHSh, −6.5 and 12·5 mV; sHHSHh, −8·8 and 10·75 mV. *C*, I_{Ba} recorded at a holding potential of −80 mV in response to 350 ms voltage steps from −30 to 20 mV in 10 mV steps.

Table 1. Functional properties of chimeric α_1 subunits

Chimera	$V_{0.5}$ (mV)	k (mV)	G_{max} (μS)	I_{Ba} increase (%)*	τ_{1act} (ms)	τ_{2act} (ms)	τ_{inact} (ms)
sH	13·6 ± 0·8	−9·2 ± 1·1	18·4 ± 6·4	200 ± 17	6·6 ± 2·1	—	198 ± 14
sHHSSs	12·2 ± 1·8	−9·3 ± 1·6	22·8 ± 10·2	150 ± 14	19·8 ± 1·8	205 ± 13·1	—
sHHSSh	9·2 ± 0·9	−9·4 ± 0·7	22·4 ± 6·45	148 ± 7	12·2 ± 1·4†	214 ± 28·7	—
sHHHSh	10·3 ± 1·3	−8·2 ± 1·3	42·2 ± 19·9	210 ± 23	12·9 ± 1·9†	262 ± 18·9	—
sHHSHh	10 ± 1·6	−8·9 ± 2·1	12·5 ± 9·4	148 ± 7	6·33 ± 1·7	—	175 ± 22

Kinetics of chimeric Ca^{2+} channels were studied during depolarizing pulses from −80 to 0 mV (see Fig. 2). Biexponential activation of sHHSSs, sHHSSh and sHHHSh and inactivation kinetics of sH and sHHSHh were studied during 800 ms voltage steps. * Increase in I_{Ba} by (−)Bay K 8644 (1 μM) was measured at 10 mV test potential. k, slope factor of steady-state activation curve; $V_{0.5}$, potential of half-maximal current activation; τ_{1act}, apparent fast activation time constant; τ_{2act}, apparent slow activation time constant; τ_{inact}, inactivation time constant. Values are means ± S.E.M. from 5–9 oocytes (3 batches). † Activation time constants τ_{1act} of chimeras sHHSSh and sHHHSh were significantly ($P < 0.05$) faster than τ_{1act} of chimera sHHSSs.

(pH adjusted to 7·4 with Tris). A membrane potential close to zero was obtained by incubating the oocytes in high potassium solution containing (mM): 150 potassium aspartate, 10 EGTA, 5 Hepes, 150 KOH (pH adjusted to 7·4 with KOH). The resting membrane potential of the oocytes in a high potassium solution was evaluated with the microelectrode technique and measured 5·6 ± 4·1 mV ($n = 4$). Data were digitized at 10 kHz in single channel studies and 2 kHz in two-microelectrode measurements and processed using the pCLAMP software 5.5 (Axon instruments, USA). Single channel data and two-microelectrode data were filtered at 1 and 0·5 kHz, respectively. All experiments were carried out at 20–22 °C. Values are calculated as means ± S.E.M. Statistical significance was evaluated by Student's t test ($P < 0.05$).

RESULTS

A comparison of the current kinetics of chimeras sHHSSs and sH revealed that I_{Ba} of sHHSSs activates with a significantly slower time course compared with currents after expression of sH (see Fig. 1). Whereas the sH current activation at 0 mV can be well described by a single exponential function and shows inactivation during a 350 ms test pulse (Fig. 2*A*), two time constants were required for the more complex activation kinetics of chimera sHHSSs which did not reach peak current during the 350 ms pulse (Fig. 2*B*). The two apparent activation time constants of chimera sHHSSs differ by a factor of 5 or more, depending on the test potential. The parameters of the steady-state voltage dependence of Ca^{2+} channel activation of the studied constructs were determined from the current–voltage relationships of I_{Ba} (see Table 1). We observed little variation in the voltage dependence of steady-state activation between different chimeric Ca^{2+} channels (Fig. 1*B*, Table 1). The kinetics of current activation of various constructs could therefore be compared at a standard test potential of 0 mV.

Comparison of Ca^{2+} channel current kinetics of sH and sHHSSs (Fig. 2) suggests the existence of structural elements

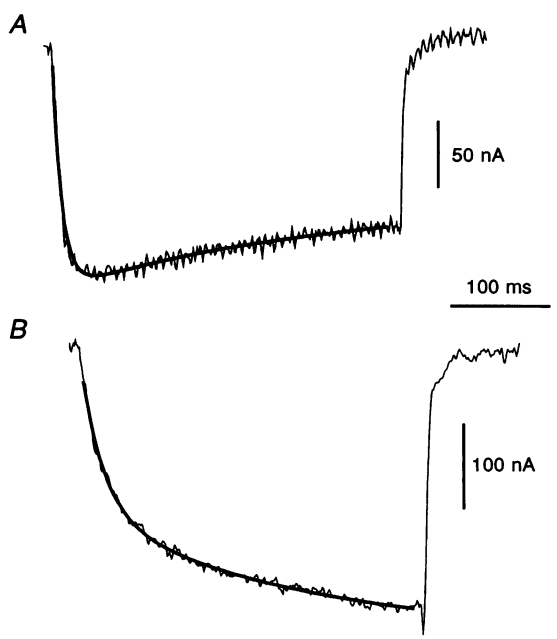

Figure 2. Current kinetics of chimeras sH and sHHSSs
I_{Ba} of chimeras sH (*A*) and sHHSSs (*B*) were elicited by 350 ms depolarizing voltage steps from −80 to 0 mV. The continuous lines represent the best fit to a double exponential function $I_{Ba}(t) = a\exp(-t/\tau_1) + b\exp(-t/\tau_2) + c$. *A*, $\tau_1 = 6.7$ ms describes a monoexponential current activation and τ_2 is the inactivation time constant $\tau_{inact} = 193$ ms approximating the current decay of chimera sH during the test pulse (see Table 1). *B*, the biexponential increase of I_{Ba} after stepping the membrane potential from −80 mV to the test potential of 0 mV is described by two activation time constants $\tau_{1act} = 19$ ms and $\tau_{2act} = 188$ ms.

in repeat III and/or IV, distinct from a previously identified sequence in repeat I (Tanabe *et al.* 1991; Nakai *et al.* 1994), which determine current activation. For a more precise localization of structural elements affecting L-type Ca^{2+} current activation we studied I_{Ba} kinetics of the constructs sHHSSh, sHHHSh and sHHSHh (see Methods). Currents of chimera sHHSSh activated with a slow and biexponential time course. Significantly faster activating I_{Ba}, also displaying inactivation, were observed in oocytes expressing chimera sHHSHh. The time course of sHHSHh activation was practically indistinguishable from the kinetics of sH (see Fig. 1 and Table 1). In contrast, expression of sHHHSh, where III S6 to IV S5 and adjacent amino acids were changed from the cardiac to the skeletal muscle sequence (note the identity of both transmembrane segments III S6 with only four different amino acids towards the amino terminus), yielded channels that activate more slowly than sHHSHh but somewhat faster than sHHSSs (see Table 1 for apparent activation time constants).

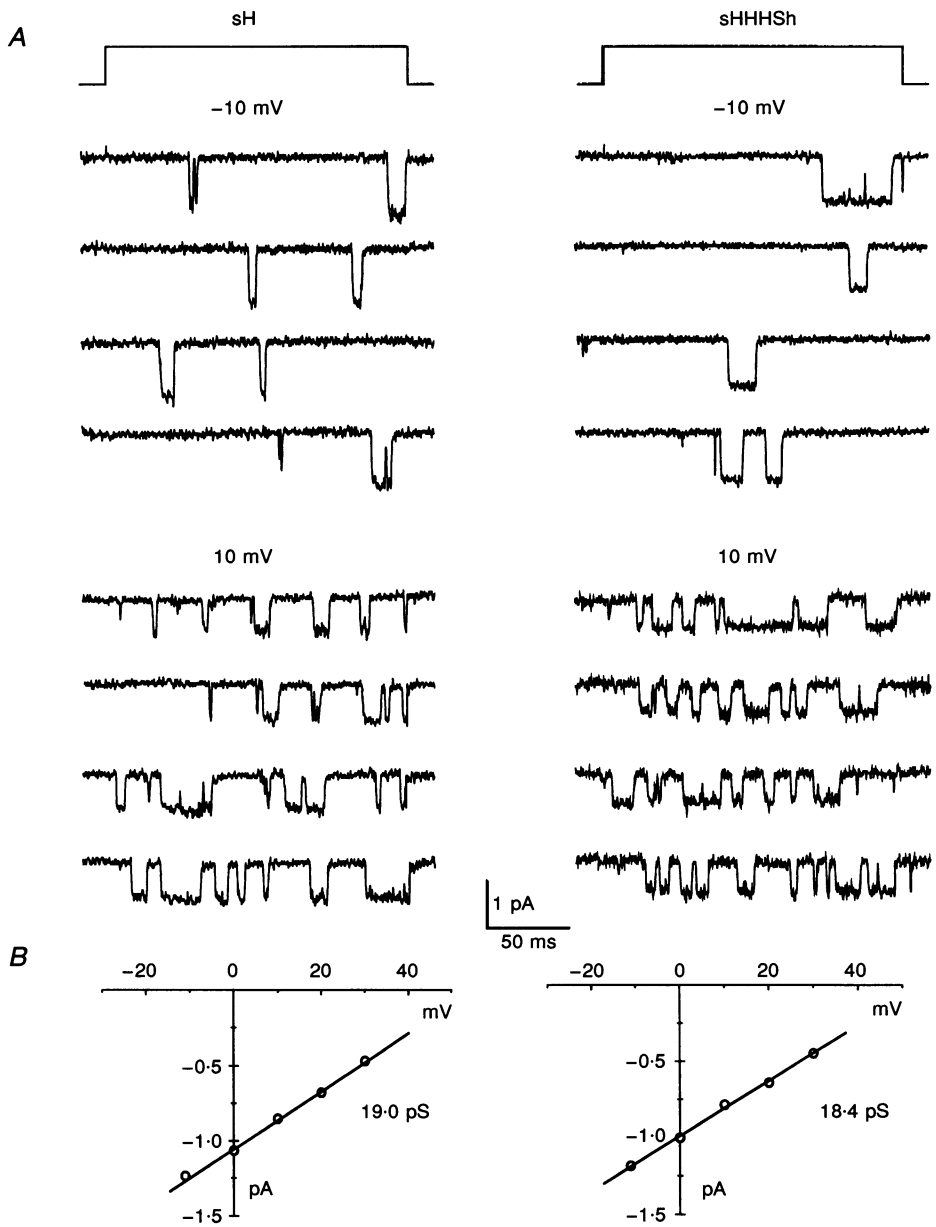

Figure 3. Single channel recordings and current–voltage relationships of unitary currents of fast and slow activating chimeras sH and sHHHSh

A, single channel recordings of chimeras sH and sHHHSh in the presence of 1 μM (−)Bay K 8644. Depolarizing pulses were applied from a holding potential of −80 mV; corresponding test potentials are indicated. *B*, current–voltage relationships of single channel currents corresponding to the records in *A*. Open channel current–voltage relationships were obtained from all point amplitude histograms with 90 mM Ba^{2+} as charge carrier. All records were filtered at 1 kHz.

Single channel studies in BC3H1 cells suggest that the biexponential activation of skeletal muscle Ca^{2+} channels is associated with two conductance states (Caffrey, 1994). We therefore performed single channel studies to investigate if the changes in macroscopic current kinetics of L-type currents were accompanied by changes in single channel properties. Figure 3 shows single channel recordings of the fast-activating chimera sH and the slowly activating chimera sHHHSh at −10 and 10 mV test potential, respectively. The experiments were performed with 1 μM (−)Bay K 8644 added to the pipette solution. Under these conditions (−)Bay K 8644 promoted long-lasting single channel openings in both chimeras which were not seen under control conditions. This finding is consistent with the observed increase of I_{Ba} after application of (−)Bay K 8644 (Table 1). The unitary conductances of single channels were calculated to be $19{\cdot}4 \pm 1{\cdot}1$ pS ($n = 4$) for sH and $18{\cdot}6 \pm 1{\cdot}2$ pS ($n = 3$) for sHHHSh.

DISCUSSION

In the present study we report first evidence for structural elements in the repeats III and IV of L-type Ca^{2+} channel α_1 subunits that determine a slow and biexponential activation of L-type Ca^{2+} channel currents. Motifs determining current activation have been identified so far in repeat I within the transmembrane segment S3 and the extracellular S3–S4 linker (Tanabe *et al.* 1991; Nakai *et al.* 1994).

Functional properties of the half–half chimera sHHSSs derived from the rapidly activating cardiac $\alpha_{1C\text{-}a}$ channel (Sather *et al.* 1993) and the carp skeletal muscle α_{1S} (Grabner *et al.* 1991) indicate that replacing sequences of repeat III and IV of the cardiac chimera sH by the corresponding sequences of α_{1S} converts the current kinetics from fast and monoexponential to slow and biexponential (see Fig. 2 and Table 1). Furthermore, the macroscopic Ca^{2+} channel currents of chimera sHHSSs did not display observable current inactivation during an 800 ms test pulse. The importance of region IIIS6–IVS5 for the kinetics of voltage-dependent activation is highlighted by slow activation of chimera sHHHSh (see Table 1).

The biexponential nature of the current activation observed in the present study in slowly activating chimeras clearly distinguishes the regulation of current activation by elements of repeat III and IV from previous results obtained from dysgenic mouse myotubes. In dysgenic mouse myotubes chimeras with skeletal muscle properties activate with monoexponential time course, in other words a single time constant is sufficient to describe current activation kinetics (Tanabe *et al.* 1991; Nakai *et al.* 1994). The current activation kinetics of slow activating chimeras described in the present study is more complex. Hence, a component of inactivation (i.e. determined by structural elements of IVS6 or the carboxy terminus) could affect the apparent activation time constants (see Table 1) and complicate the unequivocal description of the current activation process by two time constants. The longer time to peak of channel constructs possessing 'slow activation' is, however, difficult to explain by a simple removal of current inactivation as the activation rates of sH and sHHSHh were significantly faster compared with the two apparent activation time constants of chimeras sHHSSs, sHHSSh and sHHHSh (see Table 1).

In our study a modulatory influence of exogenous β, α_2-δ and γ subunits on I_{Ba} kinetics was excluded by expressing exclusively α_1 chimeras. Our experimental approach is different from the work performed on dysgenic mouse myotube cells (Tanabe *et al.* 1991; Nakai *et al.* 1994) where other channel subunits are known to be co-expressed (Knudson, Chaudhari, Sharp, Powell, Beam & Campbell, 1989) and the kinetic properties of the α_1 chimeras are therefore likely to be modulated by other subunits.

In contrast to cardiac L-type Ca^{2+} currents that have been extensively characterized in native cells and expression systems (Hess *et al.* 1985; Sather *et al.* 1993), little was known, until recently, concerning the biophysical properties of α_{1S} channels. Single channel studies in BC3H1 cells suggested that slow-current activation is associated with two subconductance levels of α_{1S} channels (Caffrey, 1994). We did not observe subconductance levels in our single channel studies in *Xenopus* oocytes and have no evidence for two conductance levels associated with the two time constants of current activation. However, more detailed single channel studies are required to explain the biexponential nature of the slowly activating chimeras observed in the present study.

The high degree of homology between α_{1S} currents observed in patch clamp studies in BC3H1 (Caffrey, 1994) and the slowly activating I_{Ba} of sHHSSs and the other slowly activating chimeras supports the idea that these constructs have common kinetic properties with native α_{1S} channels and may serve as useful models for studies of α_{1S} channel properties in *Xenopus* oocytes. Previous attempts to express the complete carp or rabbit α_{1S} subunit in *Xenopus* oocytes were not successful, indicating a common peculiarity of the two skeletal muscle α_{1S} subunits cloned so far (Tanabe *et al.* 1987; Grabner *et al.* 1991). Taken together, our data indicate that activation kinetics of Ca^{2+} channels is influenced by more than one structural element of the α_1 subunit (Nakai *et al.* 1994). It remains to be shown whether a common key element in repeat IV (as mapped in repeat I; Nakai *et al.* 1994) determines the activation properties or whether other structural elements of repeats III and IV are responsible for the 'slow activation'.

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