

Molecular Determinants of Inactivation within the I-II Linker of $\alpha 1E$ ($Ca_v2.3$) Calcium Channels

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ABSTRACT Voltage-dependent inactivation of $Ca_v2.3$ channels was investigated using point mutations in the β -subunit-binding site (AID) of the I-II linker. The quintuple mutant $\alpha 1E$ N381K + R384L + A385D + D388T + K389Q (NRADK-KLDTQ) inactivated like the wild-type $\alpha 1E$. In contrast, mutations of $\alpha 1E$ at position R378 (position 5 of AID) into negatively charged residues Glu (E) or Asp (D) significantly slowed inactivation kinetics and shifted the voltage dependence of inactivation to more positive voltages. When co-injected with $\beta 3$, R378E inactivated with $\tau_{inact} = 538 \pm 54$ ms ($n = 14$) as compared with 74 ± 4 ms ($n = 21$) for $\alpha 1E$ ($p < 0.001$) with a mid-potential of inactivation $E_{0.5} = -44 \pm 2$ mV ($n = 10$) for R378E as compared with $E_{0.5} = -64 \pm 3$ mV ($n = 9$) for $\alpha 1E$. A series of mutations at position R378 suggest that positively charged residues could promote voltage-dependent inactivation. R378K behaved like the wild-type $\alpha 1E$ whereas R378Q displayed intermediate inactivation kinetics. The reverse mutation E462R in the L-type $\alpha 1C$ ($Ca_v1.2$) produced channels with inactivation properties comparable to $\alpha 1E$ R378E. Hence, position 5 of the AID motif in the I-II linker could play a significant role in the inactivation of $Ca_v1.2$ and $Ca_v2.3$ channels.

INTRODUCTION

The influx of calcium through neuronal voltage-gated Ca^{2+} channels regulates a wide range of cellular processes, including neurotransmitter release, activation of Ca^{2+} -dependent enzymes and second messenger cascades, gene regulation, and proliferation. To this date, molecular cloning has identified the primary structures for 10 distinct calcium channel α_1 subunits: $\alpha 1S$ ($Ca_v1.1$), $\alpha 1C$ ($Ca_v1.2$), $\alpha 1D$ ($Ca_v1.3$), and $\alpha 1F$ ($Ca_v1.4$) encode L-type channels; $\alpha 1A$ ($Ca_v2.1$) encodes both P- and Q-type channels; $\alpha 1B$ ($Ca_v2.2$) defines N-type channels; $\alpha 1G$ ($Ca_v3.1$), $\alpha 1H$ ($Ca_v3.2$), and $\alpha 1I$ ($Ca_v3.3$) form T-type channels (Cribbs et al., 1998, 2000; Lee et al., 1999a; Monteil et al., 2000; Ertel et al., 2000) and $\alpha 1E$ ($Ca_v2.3$) probably encodes a component of the resistant current identified in some neuronal preparations (Randall and Tsien, 1997; Piedras-Renteria and Tsien, 1998; Saegusa et al., 2000).

Calcium channel inactivation is a critical determinant of the temporal precision of calcium signals and serves to prevent long-term increases in intracellular calcium levels. In the L-type $\alpha 1C$ channel, inactivation proceeds mostly in response to localized elevation of intracellular Ca^{2+} providing negative Ca^{2+} feedback (deLeon et al., 1995; Bernatchez et al., 1998). The dominant Ca^{2+} sensor for such Ca^{2+} -dependent inactivation in $\alpha 1C$ and $\alpha 1A$ Ca^{2+} channels has recently been identified as calmodulin, which appears to be constitutively tethered to the channel complex (Qin et al., 1999; Zuhlke et al., 1999; Peterson et al., 1999; Lee et al., 1999b). This Ca^{2+} sensor induces channel inac-

tivation by Ca^{2+} -dependent calmodulin binding to an IQ-like motif situated on the carboxyl tail of $\alpha 1C$ (Peterson et al., 2000).

Fast and voltage-dependent inactivation appears to be a key mechanism by which other Ca^{2+} channels achieve regulation of internal calcium levels. The molecular mechanisms for voltage-dependent inactivation in Ca^{2+} channel proteins are incompletely understood. The importance of the I-II linker in Ca^{2+} channel inactivation has, however, recently emerged in various studies. Point mutations and chimeras in that region were shown to modify inactivation kinetics in $\alpha 1A$ (Herlitze et al., 1997) and $\alpha 1C$ channels (Adams and Tanabe, 1997). Overexpression of mRNA coding for the I-II linker from $\alpha 1A$, but not for the III-IV linker, was shown to speed up inactivation of wild-type $\alpha 1A/\beta 2a$ in *Xenopus* oocytes (Cens et al., 1999). A chimeric channel containing the I-II linker from $\alpha 1E$ accelerated the inactivation kinetics of $\alpha 1C$ (Stotz et al., 2000). Conversely we have also recently observed that the I-II linker from $\alpha 1C$ conferred slower inactivation kinetics to $\alpha 1E$ (unpublished data). Hence, the I-II linker of HVA α_1 subunits appears a likely candidate for an inactivating blocking particle in an updated version of the hinged-lid mechanism observed in Na^+ channels. In this model, most β -subunits would regulate Ca^{2+} channel inactivation kinetics by priming the I-II linker into a conformation more favorable to inactivation.

The I-II linker contains many crucial regulatory sites in Ca^{2+} channels. The I-II linker contains the high-affinity β -subunit-binding site AID (alpha-1 subunit interaction domain) that was first identified by protein overlay (Pragnell et al., 1994) and was shown to participate in β -subunit regulation (DeWaard and Campbell, 1995; Gerster et al., 1999; DeWaard et al., 1996). Mutations within the AID motif in $\alpha 1A$ perturbed β binding (Pragnell et al., 1994). There are secondary binding sites between the cytoplasmic β - and the α_1 -subunits on the N-terminus for $\alpha 1B$ (Ste-

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phens et al., 2000) and $\alpha 1A$ (Walker et al., 1999) and on the C-terminus for $\alpha 1E$ (Tareilus et al., 1997), but AID appears to be universally present in all non-T-type $\alpha 1$ -subunits (Perez-Reyes et al., 1998). The AID binding site is composed of QQXEXXLXGYXXWIXXXE, and a single substitution at the conserved Y (Tyr) position could disrupt plasma membrane targeting of the $\alpha 1$ -subunit without, however, affecting the β -subunit-induced modulation of whole-cell and single-channel currents (Gerster et al., 1999). The I-II linker of the $\alpha 1$ -subunit contains an endoplasmic reticulum retention signal that is antagonized by the β -subunit (Bichet et al., 2000). Last, the I-II linker was also shown to interact with G-protein $\beta\gamma$ -subunit (DeWaard et al., 1997; Dolphin, 1998) in a competitive fashion with β -subunits (Campbell et al., 1995).

Recent studies have strongly suggested a critical role for the I-II linker, and more precisely for the β -subunit-binding site (AID) in the inactivation of voltage-dependent Ca^{2+} channels (VDCCs) (Page et al., 1997; Herlitze et al., 1997). Indeed, conversion of the AID motif from QQIERE to QQIEEE slowed inactivation of $\alpha 1A$ channels as well as shifting its voltage dependence of inactivation to more positive potentials (Herlitze et al., 1997).

Point mutations E462R in $\alpha 1C$ and its counterpart R387E in $\alpha 1E$ channels were herein shown to significantly influence both the kinetics and the voltage dependence of inactivation. Furthermore, a quintuple mutant made in the same region, $\alpha 1E$ N381K + R384L + A385D + D388T + K389Q, failed to affect either kinetics or voltage dependence of inactivation. Hence, along with containing the G-protein regulation site and the retention signal, the I-II loop in Ca^{2+} channel $\alpha 1$ -subunits may thus underlie three important modulatory influences in VDCCs.

MATERIALS AND METHODS

Recombinant DNA techniques

Standard methods of plasmid DNA preparation were used (Sambrook et al., 1989). cDNAs coding for wild-type rabbit $\alpha 1C$ (GenBank X15539) and $\beta 3$ (Genbank M88751) were kindly donated by Dr. E. Perez-Reyes. The wild-type human $\alpha 1E$ (GenBank L27745) was a gift from Dr. T. Schneider. The rat brain $\alpha 2b\delta$ -subunit was provided by Dr. T. P. Snutch. For the $\alpha 1C$ mutants, a site *XhoI* was first engineered by polymerase chain reaction into $\alpha 1C$ at position 1530 nt in the I-II linker, roughly 40 residues downstream to the β -subunit-binding site on the $\alpha 1$ -subunit (AID). This is a nonsilent mutation creating a Gly-to-Arg mutation at this position. The resulting $\alpha 1C$ (*XhoI*) channel, however, displayed inactivation and activation kinetics similar to the wild-type $\alpha 1C$ (see Figs. 2 and 7). For point mutations, repeat I of $\alpha 1E$ and $\alpha 1C$ were respectively subcloned in Topo XL (Invitrogen, Carlsbad, CA) for conveniently using the Unique Site Elimination method (Pharmacia Biotech) developed by Deng and Nickoloff (1992) with *DraI*/*HpaI* selection. Constructs were verified by restriction mapping, and recombinant clones were screened by double-stranded sequence analysis of the entire ligated cassette. The nucleotide sequence of the mutated region was determined by the dideoxy chain termination method using either single- or double-stranded plasmid DNA (T7 Sequenase v 2.0, Amersham Pharmacia Biotech) in house or sent out for automatic sequencing by

BioST (Lachine, Québec, Canada). cDNA constructs for wild-type and mutated $\alpha 1$ -subunits were linearized at the 3' end by *HindIII* digestion whereas the rat brain $\beta 3$ -subunit was digested by *NotI*. Run-off transcripts were prepared using methylated cap analog m⁷G(5')ppp(5')G and T7 RNA polymerase with the mMessage mMachine transcription kit (Ambion, Austin, TX). The final cRNA products were resuspended in DEPC-treated H₂O and stored at -80°C . The integrity of the final product and the absence of degraded RNA was determined by a denaturing agarose gel stained with ethidium bromide.

Functional expression of wild-type and mutant channels

Oocytes were obtained from female *Xenopus laevis* clawed frog (Nasco, Fort Atkinson, WI) as described previously (Parent et al., 1995, 1997; Parent and Gopalakrishnan, 1995; Bernatchez et al., 1998). Individual oocytes free of follicular cells were obtained after 30–40 min of incubation in a calcium-free solution (in mM: 82.5 NaCl, 2.5 KCl, 1 MgCl₂, 5 Hepes, pH 7.6) containing 2 mg/ml collagenase (Gibco, Burlington, Ontario, Canada). Forty-six nanoliters of a solution containing between 35 and 50 ng of cRNA coding for the wild-type or mutated $\alpha 1$ -subunit was injected 16 h later into stage V and VI oocytes. The cRNA concentration of the $\alpha 1$ -subunit was generally adjusted to yield whole-cell peak currents in the 1–5- μA range; hence RNA concentration coding for the $\alpha 1E$ wild-type and mutant channels was established at the lowest end of this range whereas $\alpha 1C$ wild-type and $\alpha 1C$ E462R channels were measured after injection with the highest concentration possible. When specified, cRNA coding for rat brain $\alpha 2b\delta$ (Williams et al., 1992) and rat brain $\beta 3$ (Castellano et al., 1993) were co-injected with the $\alpha 1$ -subunit at a 3:1:1 weight ratio. Oocytes were incubated at 19°C in a Barth's solution (in mM): 100 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, 2.5 pyruvic acid, 100 U/ml penicillin, 50 $\mu\text{g}/\text{ml}$ gentamicin, pH 7.6. The inactivation properties of each mutant channel herein described was studied in a minimum of three different oocyte batches to account for the typical expression variability encountered from batch to batch. Furthermore, the corresponding wild-type channel ($\alpha 1E$ in most cases) was systematically injected under the same experimental conditions every time a new mutant was analyzed, thus insuring that the mutant behavior was not caused by some aberrant properties (such as higher level of endogenous β -subunits) (Lacerda et al., 1994; Tareilus et al., 1997) of this particular batch of oocytes. Hence the properties of the wild-type $\alpha 1E$ channel were analyzed in seven different oocyte batches for the current study.

Electrophysiological recordings in oocytes

Wild-type and mutant channels were screened at room temperature for macroscopic barium current 4 to 7 days after RNA injection using a two-electrode voltage-clamp amplifier (OC-725C, Warner Instruments, Hamden, CT) as described earlier (Parent et al., 1995, 1997). Voltage and current electrodes were slightly broken under the microscope to decrease the electrode resistance to 0.5–1.5-M Ω tip resistance and were filled with 3 M KCl, 1 mM EGTA, 10 mM HEPES (pH 7.4). Oocytes were first impaled in a modified Ringer solution (in mM): 96 NaOH, 2 KOH, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES titrated to pH 7.4 with methanesulfonic acid CH₃SO₃H (MeS). The bath was then perfused with the 10 mM Ba²⁺ solution (in mM: 10 Ba(OH)₂, 110 NaOH, 1 KOH, 20 Hepes titrated to pH 7.3 with MeS). To minimize kinetic contamination by the endogenous Ca²⁺-activated Cl[−] current, oocytes were injected with 18.4 nl of a 50 mM EGTA solution (Sigma, St. Louis, MO) 0.5–2 h before the experiments. Oocytes were superfused by gravity flow at a rate of 2 ml/min, which was fast enough to allow complete chamber fluid exchange within 30 s. Experiments were performed at room temperature (20–22°C).

Data acquisition and analysis

PCLamp software, Clampex 6.02 and Clampfit 6.02 (Axon Instruments, Foster City, CA), was used for on-line data acquisition and analysis. Unless stated otherwise, data were sampled at 10 kHz and low pass filtered at 5 kHz using the amplifier built-in filter. For all recordings, a series of voltage pulses were applied from a holding potential of -80 mV at a frequency of 0.2 Hz from -40 to $+60$ mV. Isochronal inactivation data (h_∞ or h_{inf}) were obtained from tail currents generated at the end of a 5-s prepulse (Parent et al., 1995). Tail current amplitudes were estimated using the function Analyze in Clampfit 6.0 from the peak current arising during the first 10 ms after the capacitive transient (20 data points). Each of these currents was then normalized to the maximum current obtained before the prepulse voltage (i/i_{max}) and was plotted against the prepulse voltage. For the isochronal inactivation figures, data points represent the mean of $n \geq 3$ and were fitted to the Boltzmann Eq. 1:

$$\frac{i}{i_{max}} = 1 - \frac{1 - Y_0}{1 + \left\{ \exp - \frac{zF}{RT} (V_m - E_{0.5}) \right\}} \quad (1)$$

Pooled data points (mean \pm SEM) were fitted to Eq. 1 using user-defined functions and the fitting algorithms provided by Origin 6.0 (Microcal Software, Northampton, MA) analysis software. Eq. 1 accounts for the fraction of non-inactivating current with $E_{0.5}$, mid-point potential; z , slope parameter; Y_0 , fraction of non-inactivating current; V_m , the prepulse potential; and RT/F with their usual meanings. The fitting process generated values estimating errors on the given fit values.

Activation parameters were estimated from the mean I - V curves obtained for each channel combination. The I - V relationships were normalized to the maximum amplitude and were fitted to the Boltzmann Eq. 2:

$$\frac{i}{i_{max}} = \frac{1}{1 + \left\{ \exp - \frac{zF}{RT} (V_m - E_{0.5}) \right\}} \quad (2)$$

$E_{0.5}$ is the potential for 50% activation, z is slope parameter, V_m is the test potential, and RT/F have their usual meanings. The fitting process generated values estimating errors on the given fit values.

As the number of exponential functions needed to account for the inactivation process varied between $\alpha 1C$ and $\alpha 1E$ channels, inactivation kinetics were quantified using r300 values, that is, the ratio of the whole-cell current remaining at the end of a 300-ms pulse. As inactivation kinetics can vary with current density, comparisons between constructs and mutants were generally restricted to whole-cell currents lower than $5 \mu A$ as much as possible. Furthermore, this range of current densities made it easier to voltage clamp the oocyte uniformly, thus decreasing the possibility of series resistance artifacts contaminating the current kinetics data.

Capacitive transients were erased for clarity in the final figures. Statistical analyses and Student t -test were performed using the fitting routines provided by Origin 5.0 and 6.0 (Microcal Software).

RESULTS

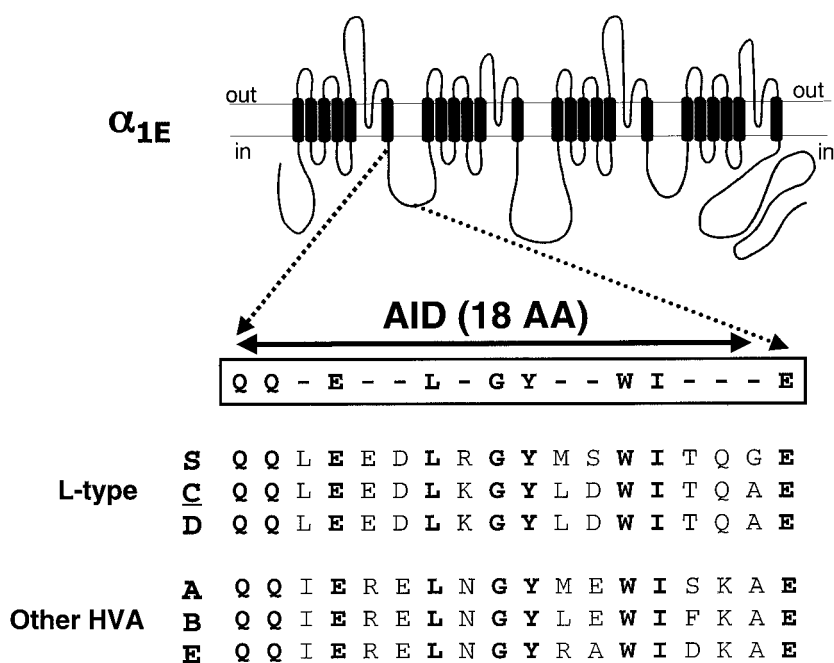
Recent studies have strongly suggested a critical role for the I-II linker and, more precisely, for the β -subunit-binding site (AID) in the inactivation of voltage-dependent Ca^{2+} channels (VDCCs) (Page et al., 1997; Herlitze et al., 1997; Adams and Tanabe, 1997; Cens et al., 1999; Stotz et al., 2000). Some unpublished results from our lab obtained using $\alpha 1E/\alpha 1C$ chimeras also point toward the high-affinity β -subunit-binding site in the I-II of the $\alpha 1$ -subunit (AID) as

an important determinant of voltage-dependent inactivation in $\alpha 1E$ channels (unpublished data). As the AID motif is conserved in all non-T-type $\alpha 1$ -subunits, this observation suggests a role for nonconserved residues present in the AID domain. It is composed of a short stretch of 18 residues located in the 5' end of the I-II linker. Fig. 1 shows the consensus sequence QQXEXXXLGYXXWIXXXE for the β -subunit-binding site in the six gene families $\alpha 1S$, $\alpha 1C$, $\alpha 1D$, $\alpha 1A$, $\alpha 1B$, and $\alpha 1E$. Identical residues are shown in bold. Of the nine nonconserved residues, positions 3 and 6 of the AID motif involve conservative mutation from an Iso (I) to a Leu (L) and from a Glu (E) to an Asp (D); position 17 is actually a conserved Ala (A) residue between $\alpha 1E$ and $\alpha 1C$. Position 5 is occupied by an Arg (R) that is strictly conserved in non-L-type and is replaced by a Glu (E) in L-type. Hence we undertook a detailed mutagenesis study of the six nonconservative residues in the AID motif of $\alpha 1E$ with R378, N381, R384, A385, D388, or K389 alone or in combination.

Residue R378 is critical for $\alpha 1E$ (Ca_v2.3) fast inactivation kinetics

Fig. 2 shows a family of whole-cell current recordings obtained in the presence of 10 mM Ba^{2+} for the wild-type $\alpha 1E$, triple-mutant N381K + R384L + A385D, quintuple-mutant N381K + R384L + A385D + D388T + K389Q, and point mutations K389E and R378E expressed in *Xenopus* oocytes on the $\alpha 2b\delta$ and $\beta 3$ auxiliary subunit background. Current traces recorded under the same conditions for the modified $\alpha 1C$ (*XhoI*) channel (used to produce the $\alpha 1C$ E462R mutant) are shown for comparison. The $\beta 3$ -subunit was the chosen subunit as it triggers inactivation mostly from the closed state in neuronal Ca^{2+} channels as opposed to $\beta 2a$ that favors inactivation from the open state (Patil et al., 1998). The quintuple-mutant NRADK-KLDTQ includes five of the six nonconserved residues between $\alpha 1E$ and $\alpha 1C$ in the AID motif. As seen, the triple-mutant NRA-KLD (positions 8, 11, and 12 of AID) and the quintuple-mutant NRADK-KLDTQ (positions 8, 11, 12, 15, and 16 of AID) inactivated like the wild-type $\alpha 1E$ channel. Similar data were obtained with the quadruple-mutant NRAK-KLDQ (positions 8, 11, 12, and 16 of AID; results not shown). For instance, $95 \pm 1\%$ ($n = 21$) of the $\alpha 1E$ whole-cell currents were inactivated at the end of a 300-ms pulse to 0 mV, which is identical to $96 \pm 1\%$ ($n = 3$) for NRA-KLD, $96 \pm 1\%$ ($n = 4$) for NRAK-KLDQ, and $96 \pm 2\%$ ($n = 5$) for NRADK-KLDTQ. In contrast, a single point mutation at position R378 (position 5 in AID) produced whole-cell currents with significantly slower inactivation kinetics with only $72 \pm 4\%$ ($n = 13$) of the R378E currents being inactivated under the same conditions. Mutation of the positively charged Lys residue at position 389 (position 16 in AID) to a negatively charged Glu (K to E) produced an intermediary inactivation phenotype with $85 \pm 2\%$ ($n =$

FIGURE 1 Predicted secondary structure for the human brain $\alpha 1E$ ($Ca_v2.3$) channel with the four homologous repeats and the N and the C termini facing the cytoplasm. The β -subunit-binding site on the $\alpha 1$ subunit (AID) is located within 20 residues of the IS6 transmembrane segment. The consensus sequence for the AID motif (QQxExxLxGYxxWlxxxE) is shown with conserved residues in bold letters. Non-conserved residues are represented by slashes (-) on the figure. The amino acid alignment for L-type ($\alpha 1S$ ($Ca_v1.1$), $\alpha 1C$ ($Ca_v1.2$), and $\alpha 1D$ ($Ca_v1.3$)) and non-L-type ($\alpha 1A$ ($Ca_v2.1$), $\alpha 1B$ ($Ca_v2.2$), and $\alpha 1E$ ($Ca_v2.3$)) Ca^{2+} channels within this region is displayed enlarged. Of the nine "x" sites, positions 3 and 6 of the AID involve conservative mutation from an Iso (I) to a Leu (L) and from a Glu (E) to an Asp (D); position 17 is actually a conserved Ala (A) residue between $\alpha 1E$ and $\alpha 1C$. Position 5 of AID is occupied by an Arg (R) that is strictly conserved in non-L-type and is replaced by a Glu (E) in L-type. These residues correspond to R378 in $\alpha 1E$ and E462 in $\alpha 1C$.



15) of the currents inactivated at the end of a pulse to 0 mV. It should be noted that the milder K389Q mutation achieved within the quadruple and the quintuple mutants produced no significant effect on inactivation (Fig. 2). These rates of inactivation remained significantly faster than the rates observed for $\alpha 1C$ (*XhoI*), for only $29 \pm 3\%$ ($n = 6$) of these currents were inactivated under the same conditions. Current density did not appear to play a significant role in

modulating inactivation kinetics in this series of experiments (Table 1). Whole-cell current density was lower for the fast inactivating mutant $\alpha 1E$ NRA-KLD whereas the largest currents were generally recorded for $\alpha 1E$ R378E.

Inactivation kinetics were computed using r300 ratios (Peterson et al., 2000), i.e., the fraction of whole-cell currents remaining at the end of a 300-ms pulse (Fig. 3 A). As seen, the rate of inactivation increased slightly with depo-

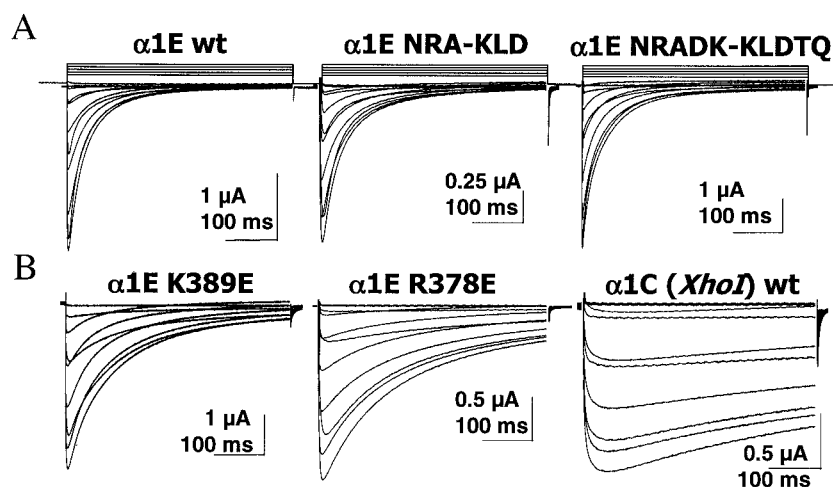


FIGURE 2 The $\alpha 1E$ wt, $\alpha 1E$ N381K + R384L + A385D (NRA), and $\alpha 1E$ N381K + R384L + A385D + D388T + K389Q (NRADK) (A) and $\alpha 1E$ K389E, $\alpha 1E$ R378E, and $\alpha 1C$ (*XhoI*) (B) were expressed in *Xenopus* oocytes in the presence of $\alpha 2b\delta$ and $\beta 3$ subunits. Whole-cell currents were recorded using the two-electrode voltage-clamp technique in the presence of 10 mM Ba^{2+} after injection of EGTA. Holding potential was -80 mV. Oocytes were pulsed from -40 mV to $+60$ mV using 10-mV steps for 450 ms. Capacitive transients were erased for the first millisecond after the voltage step. All mutants tested expressed significant whole-cell currents. Inactivation kinetics were slower in mutants K389E and R378E. Mutant and wild-type channels displayed similar activation properties (Table 1). Whole-cell currents peaked at -4 ± 2 mV ($n = 7$) for $\alpha 1E$; -3 ± 3 mV ($n = 3$) for NRA-KLD; -5 ± 3 mV ($n = 4$) for NRAK-KLDQ; -3 ± 3 mV ($n = 3$) for NRADK-KLDTQ; -3 ± 2 mV ($n = 9$) for $\alpha 1E$ R378E, and -4 ± 2 mV ($n = 9$) for $\alpha 1C$ (*XhoI*).

TABLE 1 Biophysical properties of $\alpha 1E$ (Ca v 2.3) and $\alpha 1C$ (Ca v 1.2) channels and mutants

Channels expressed with $\alpha 2b\delta/\beta 3$	Inactivation (5 s)		Activation		Peak I_{Ba} (μA)
	$E_{0.5}$ (mV)	z	$E_{0.5}$ (mV)	z	
$\alpha 1E$ wt	-64 ± 3 (9)	3.5 ± 0.4	-18 ± 2 (9)	6.1 ± 0.4	-3.7 ± 1.3 (21)
$\alpha 1E$ NRA-KLD	-68 ± 2 (7)	2.8 ± 0.5	-15 ± 2 (7)	6.0 ± 0.5	-1.9 ± 0.4 (7)
NRAK-KLDQ	-64 ± 3 (4)	3.1 ± 0.5	-11 ± 2 (4)	5.7 ± 0.4	-3.6 ± 1.4 (4)
NRADK-KLDTQ	-60 ± 2 (3)	2.8 ± 0.6	-15 ± 1 (3)	5.8 ± 0.2	-2.4 ± 1.2 (5)
$\alpha 1E$ K389E	-60 ± 3 (5)	2.2 ± 0.7	-14 ± 4 (9)	5.4 ± 0.2	-4.1 ± 1.1 (15)
$\alpha 1E$ R378K	-63 ± 2 (5)	3.2 ± 0.3	-14 ± 3 (5)	4.8 ± 0.3	-2.7 ± 1.5 (7)
$\alpha 1E$ R378A	-51 ± 2 (6)*	3.3 ± 0.2	-21 ± 5 (6)	5.1 ± 0.3	-2.6 ± 0.6 (7)
$\alpha 1E$ R378Q	-51 ± 2 (8)*	3.1 ± 0.2	-12 ± 3 (8)	4.8 ± 0.3	-3.1 ± 0.5 (8)
$\alpha 1E$ R378G	-52 ± 2 (10)*	2.9 ± 0.4	-19 ± 4 (11)	6.1 ± 0.2	-3.8 ± 1.0 (11)
$\alpha 1E$ R378D	-46 ± 3 (11) [†]	2.7 ± 0.3	-16 ± 3 (9)	6.2 ± 0.5	-3.5 ± 0.7 (14)
$\alpha 1E$ R378E	-44 ± 2 (10) [†]	2.3 ± 0.5	-14 ± 3 (14)	5.5 ± 0.3	-4.6 ± 1.7 (14)
$\alpha 1C$ E462R	-31 ± 2 (12) [†]	4.2 ± 0.2	-14 ± 4 (8)	6.7 ± 0.5	-2.0 ± 0.4 (12)
$\alpha 1C$ (<i>Xhol</i>)	-23 ± 3 (8)*	3.4 ± 0.4	-13 ± 5 (8)	9 ± 2	-3.3 ± 0.3 (9)
$\alpha 1C$ wt	-20 ± 4 (12)*	3.1 ± 0.4	-11 ± 3 (7)	10 ± 2	-3.9 ± 0.5 (17)

Biophysical parameters of $\alpha 1E$ and $\alpha 1C$ wild-type (wt) and mutant channels expressed in *Xenopus* oocytes in the presence of $\alpha 2b\delta$ and $\beta 3$ subunits. Whole-cell currents were measured in 10 mM Ba $^{2+}$ throughout. The voltage dependence of inactivation was determined after 5-s pulses from -100 to $+50$ mV as shown on Fig. 6. Relative currents were fitted to Boltzmann Eq. 1. Activation data were estimated from the mean $I-V$ relationships and fitted to Boltzmann Eq. 2. Peak I_{Ba} was determined from $I-V$ relationships for the corresponding experiments. The data are shown with the mean \pm SEM and the number of samples (n) appears in parentheses. Significance of difference between $\alpha 1E$ and other channels is given by * $p < 0.05$, [†] $p < 0.005$, and $^{\ddagger}p < 0.001$.

larization for the $\alpha 1E$ wild-type and mutant channels. $\alpha 1E$ NRA-KLD, NRAK-KLDQ (results not shown), and NRADK-KLDTQ mutants behaved like the wild-type $\alpha 1E$ at all potentials tested. In contrast, the r300 ratios were significantly higher for $\alpha 1E$ R378E ($p < 0.002$) and $\alpha 1E$ K389E ($p < 0.05$) as compared with $\alpha 1E$. Altogether, these results suggest that fast inactivation in $\alpha 1E$ depends on the nature of residues at positions 5 and 16 of the AID.

The activation potentials were estimated from the mean peak current-voltage relationships (Fig. 3 *B*). Activation potentials were similar for NRA-KLD, NRADK-KLDTQ (results not shown), and $\alpha 1E$ wt with $E_{0.5}$ values around -17 mV and slightly more positive for the quadruple-mutant NRAK-KLDQ with $E_{0.5} = -11$ mV (see Table 1 for the exact values). Whereas inactivation kinetics were significantly slower for $\alpha 1E$ K389E and R378E, both mutants were found to activate in a range of potentials not significantly different than $\alpha 1E$ wt (Table 1).

Position 5 in the AID motif influences the voltage dependence of $\alpha 1E$ (Ca v 2.3) inactivation

If inactivation kinetics reflect the rate of transition to the inactivated state(s), the isochronal inactivation data provide the voltage range where channels are most likely to inactivate. The voltage dependence of inactivation was assessed from the relative tail currents measured after a series of 5-s depolarizations. Fig. 3 *C* shows a family of isochronal inactivation data for the wild-type $\alpha 1E$ channel, NRA-KLD, NRAK-KLDQ (results not shown), NRADK-KLDTQ, K389E, R378E, and $\alpha 1C$ wt. Mid-potentials of inactivation ($E_{0.5}$) were estimated from Boltzmann fits (Eq. 1). The

voltage dependence of the isochronal inactivation for the first four mutant channels were comparable, with $E_{0.5}$ varying from -60 mV (NRADK and K389E) to -68 mV (NRA). These values were not significantly different from the $E_{0.5}$ of -64 mV for wild-type $\alpha 1E$ channels (Table 1). Hence, most mutants in the AID motif inactivated in the same voltage range as the wild-type $\alpha 1E$ channel. Only mutant R378E experienced a significant shift in its voltage dependence of inactivation toward more positive potentials as compared with the wild-type $\alpha 1E$ with a $E_{0.5} = -44$ mV. This change in the voltage dependence of inactivation of $\alpha 1E$ R378E occurred without any significant shift in the voltage dependence of activation (Table 1).

Structural requirements for fast inactivation kinetics at position 378 of $\alpha 1E$ (Ca v 2.3)

Of the six nonconserved residues in the AID motif, only R378 was shown to affect both the kinetics and voltage dependence of inactivation. A series of point mutations was thus undertaken to evaluate the importance of the charge, the volume, and the hydrophilicity of the residue 378 in the $\alpha 1E$ inactivation process. Fig. 4 shows the family of whole-cell Ba $^{2+}$ current traces of mutants R378K, R378Q, R378A, R378G, R378D, and R378E after expression in *Xenopus* oocytes. Mutant $\alpha 1E$ R378K displayed the fastest inactivation kinetics closely followed by R378A and R378Q. At -10 mV, the rate of inactivation ranked as follows (from the fastest to the slowest) $\alpha 1E$ wt \approx R378K \approx R378A $>$ R378Q \gg R378G \approx R378D \approx R378E as seen by the r300 analysis shown in Fig. 5 *A*. This ranking remained true at voltages between -10 and $+20$ mV, although inactivation

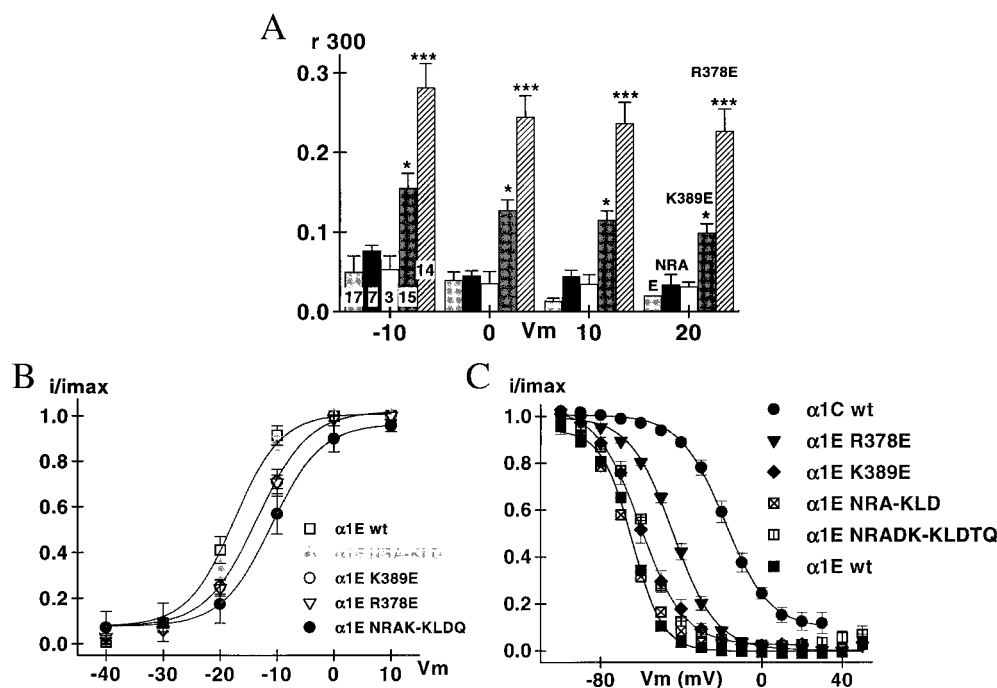


FIGURE 3 (A) The mean r300 ratios (the fraction of the whole-cell current remaining at the end of a 300-ms pulse) are shown \pm SEM at four voltages from -10 to $+20$ mV for $\alpha 1E$ wt (light gray), $\alpha 1E$ N381K + R384L + A385D (NRA) (black), $\alpha 1E$ N381K + R384L + A385D + D388T + K389Q (NRADK) (white), $\alpha 1E$ K389E (dark gray), and $\alpha 1E$ R378E (hatched) from left to right as measured in 10 mM Ba^{2+} . The numbers on the columns refer to the numbers of experiments (n) used for statistical analysis. The r300 ratios varied from 0.05 ± 0.02 at -10 mV to 0.02 ± 0 ($n = 17$) at $+20$ mV for $\alpha 1E$ and from 0.05 ± 0.02 at -10 mV to 0.03 ± 0.01 ($n = 14$) at $+20$ mV for NRADK-KLDTQ, NRAK-KLDQ, and NRA-KLD mutants when the data are pooled together. In contrast, r300 ratios were significantly different between $\alpha 1E$ wt and $\alpha 1E$ K389E ($p < 0.01$) varying from 0.16 ± 0.02 at -10 mV to 0.10 ± 0.01 ($n = 15$) at $+20$ mV for $\alpha 1E$ K389E. The r300 ratios were also significantly different between $\alpha 1E$ wt and $\alpha 1E$ R378E ($p < 0.002$) with values from 0.33 ± 0.04 at -10 mV to 0.27 ± 0.03 ($n = 14$) at $+20$ mV for R378E. (B) Activation potentials were estimated from the mean normalized current-voltage relationships. The relative data points were plotted against the test voltage and were fitted to Boltzmann Eq. 2. The activation potentials were comparable for $\alpha 1E$ wt, NRA-KLD, NRAK-KLDQ, NRADK-KLDTQ (results not shown), $\alpha 1E$ K389E, and $\alpha 1E$ R378E. The fit values are given in Table 1. (C) The voltage dependence of inactivation was measured after a 5-s conditioning prepulse applied between -100 and $+50$ mV. The protocol is shown in detail in Fig. 6. The voltage dependence of inactivation was not significantly different for $\alpha 1E$ wt, NRA-KLD, NRAK-KLDQ (results not shown), NRADK-KLDTQ, and $\alpha 1E$ K389E with $E_{0.5}$ varying from -68 to -60 mV. In contrast, the mid-potential of inactivation for R378E was -44 ± 2 mV ($n = 10$). Fit values are shown in Table 1.

kinetics tended to get faster with membrane depolarization especially for mutants R378Q, R378G, R378D, and R378E. The rate of R378K inactivation was comparable to $\alpha 1E$ at -10 and 0 mV but differed slightly at higher membrane potential as its inactivation kinetics remained relatively insensitive to depolarization. For R378Q, the r300 ratio was significantly ($p < 0.05$) different from R378K at -10 mV, but these differences were attenuated at $+20$ mV. The r300 ratios for the neutral residue R378A were noticeably similar to R378K and to the wild-type $\alpha 1E$ channel at all membrane potentials. R378A, a small and neutral residue, behaved like R378K, but R378G, which is also a small and neutral residue, behaved like negatively charged mutants R378D and R378E. Altogether, these results suggest that positively charged residues at this position may stimulate faster inactivation kinetics whereas negatively charged residues could trigger slower rates of inactivation. However, the effects of the neutral residues (A, Q, and G) cannot be simply explained in terms of either charge or size. Furthermore, none

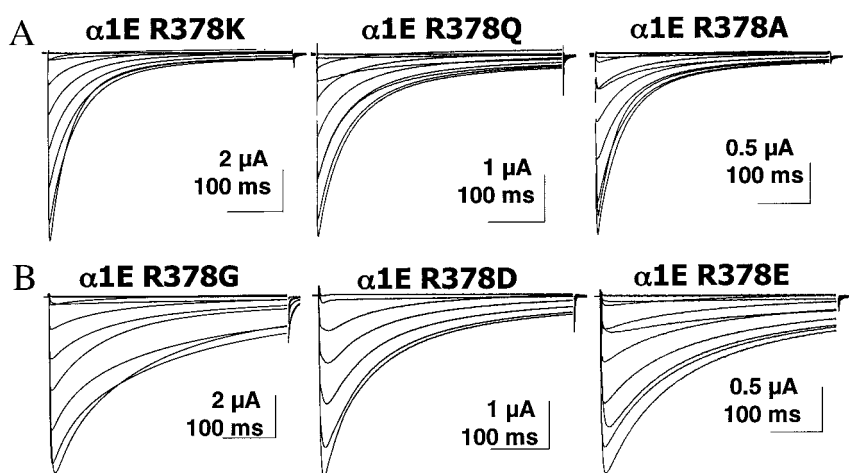
of the point mutations cause the $\alpha 1E$ channel to inactivate in the same voltage range as the wild-type $\alpha 1C$.

The slower inactivation kinetics for R378 mutants were not correlated with any apparent change in their activation kinetics. Furthermore, the activation potentials $E_{0.5}$ for mutants R378E, R378D, R378G, R378A, and R378K were comparable to the $E_{0.5}$ for the wild-type $\alpha 1E$ channel. Only R378Q appeared to activate at membrane potentials slightly more positive than the wild-type $\alpha 1E$ channel. Hence, the reported shifts in the inactivation potentials for the R378 mutants were probably not linked to changes in whole-cell activation properties.

Mutations at position 378 affect voltage-dependent inactivation of the human $\alpha 1E$ ($Ca_v2.3$)

The next series of experiments was undertaken to evaluate whether the charge of the side-chain at position 378 played

FIGURE 4 Mutants $\alpha 1E$ R378K, $\alpha 1E$ R378Q, and $\alpha 1E$ R378A (A) and $\alpha 1E$ R378G, $\alpha 1E$ R378D, and $\alpha 1E$ R378E (B) were expressed in *Xenopus* oocytes in the presence of $\alpha 2\beta\delta$ and $\beta 3$ subunits. Current traces were obtained in the presence of 10 mM Ba^{2+} after injection of EGTA. Holding potential was -80 mV. Oocytes were pulsed from -40 mV to $+60$ mV using 10-mV steps for 450 ms. Capacitive transients were erased for the first millisecond after the voltage step. All mutants tested expressed significant whole-cell currents. Inactivation kinetics appeared slower in mutants R378G, R378D, and R378E.



a role in the voltage range where $\alpha 1E$ channels inactivate. Fig. 6 shows the isochronal inactivation data measured after 5-s pulses were applied from -100 mV to voltages between -100 and $+50$ mV. Typical current traces are shown for the wild-type $\alpha 1E$ and mutants R378K, R378Q, and R378E with an example of the voltage protocol used. The inactivation data for $\alpha 1E$ R378K superimposed quite closely with the inactivation data points for the wild-type $\alpha 1E$ (Table 1). In contrast, inactivation data points are shifted to the right for mutants $\alpha 1E$ R378A, R378Q, R378G, R378D, and R378E and lay halfway between $\alpha 1C$ and $\alpha 1E$. Mid-potentials of inactivation ranged from $E_{0.5} = -52$ mV for R378G to $E_{0.5} = -44$ mV for R378E. As seen above, the shifts in the voltage dependence of inactivation were not accompanied by any significant shift in the activation potentials (Table 1).

The reverse mutation E462R triggers faster inactivation kinetics in $\alpha 1C$ ($Ca_v1.2$)

The alignment shown in Fig. 1 pointed out that the positively charged arginine (R) residue in non-L-type Ca^{2+} channels is replaced by a glutamate (E) residue that is conserved in all L-type channels. Replacement of residue 378 with the corresponding residue of $\alpha 1C$ was shown to slow inactivation kinetics and shift the voltage dependence of inactivation. The reverse mutation of E to R in the brain $\alpha 1C$ channel has already been examined and was actually shown to speed up inactivation of the slower $\alpha 1C$ channel (Herlitz et al., 1997). To compare the inactivation properties of $\alpha 1E$ R378E and $\alpha 1C$ E462R under our experimental conditions, we performed the $\alpha 1C$ E462R mutation and expressed it in *Xenopus* oocytes. Fig. 7 A shows whole-cell current traces for $\alpha 1E$, $\alpha 1C$ E462R, $\alpha 1E$ R378E, and $\alpha 1C$ that were scaled and superimposed at membrane potentials between -10 and $+10$ mV. As seen on Fig. 7 B, $\alpha 1C$ E462R and $\alpha 1E$ R378E inactivated with a similar time course that turned out to be intermediary between the fast

$\alpha 1E$ and the slow $\alpha 1C$ channel at all voltages with r300 ratios of ~ 0.3 in both cases. In contrast, r300 ratios for $\alpha 1C$ wt and $\alpha 1C$ (*XhoI*) are higher with values of ~ 0.7 . As seen, the inactivation kinetics for the modified $\alpha 1C$ (*XhoI*) channel used for making $\alpha 1C$ E462R were compiled and were found to be similar to the wild-type $\alpha 1C$ channel. Hence, neither point mutation in $\alpha 1E$ or $\alpha 1C$ could completely reverse the inactivation phenotype to the opposite wild-type channel. As inactivation kinetics of $\alpha 1C$ are exquisitely sensitive upon the current density, it should be pointed out that the current density for $\alpha 1C$ E462R was in average smaller than for $\alpha 1C$ (*XhoI*), ruling out current density as a critical factor for the faster inactivation kinetics (Table 1).

The voltage dependence of inactivation was next studied for mutant $\alpha 1C$ E462R (5-s prepulses) and compared with $\alpha 1E$, $\alpha 1E$ R378E, and $\alpha 1C$ (*XhoI*) (Fig. 7 C). As compared with the $\alpha 1C$ (*XhoI*) channel, the inactivation data points for $\alpha 1C$ E462R were shifted to the left by -10 mV, but they remained significantly more positive than the inactivation curve for $\alpha 1E$ R378E. Furthermore, the slope of the fit was much steeper for $\alpha 1C$ E462R than for any other mutant tested in this study.

Given the fact that the I-II linker could also be involved in calcium-dependent inactivation (Adams and Tanabe, 1997), the inactivation kinetics of $\alpha 1C$ E462R were also measured in the presence of 10 mM Ca^{2+} . Like the wild-type $\alpha 1C$, $\alpha 1C$ E462R was found to inactivate significantly faster in the presence of Ca^{2+} ions. For instance, the r300 ratios went from 0.39 ± 0.04 ($n = 6$) in Ba^{2+} to 0.14 ± 0.01 ($n = 3$) in Ca^{2+} at -10 mV and from 0.26 ± 0.02 ($n = 6$) in Ba^{2+} to 0.014 ± 0.001 ($n = 3$) in Ca^{2+} at $+20$ mV. These numbers compare well with the r300 ratios for $\alpha 1C$ under the same Ca^{2+} conditions with 0.13 ± 0.03 at -10 mV and 0.14 ± 0.01 at $+20$ mV ($n = 8$). This result indicates that mutations in the AID motif that disrupt voltage-dependent inactivation may not necessarily impair calcium-dependent inactivation, and further suggests that AID may not be the blocking particle common to both voltage-

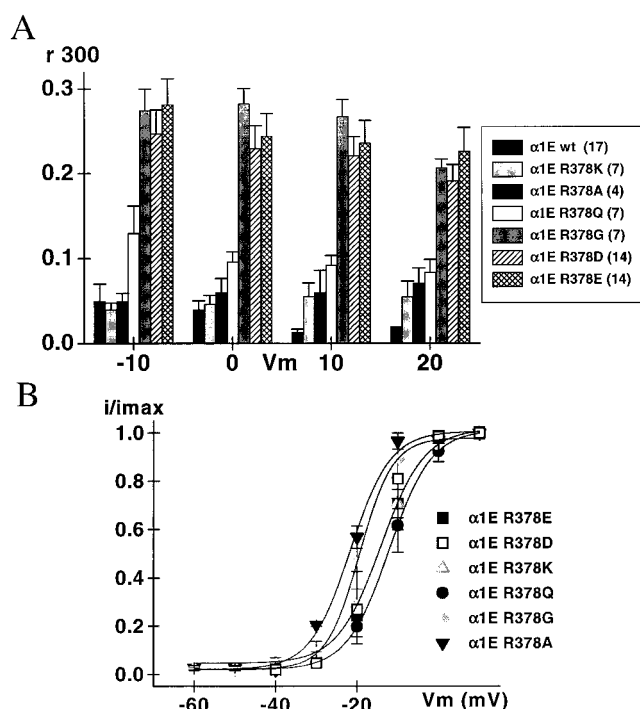


FIGURE 5 (A). Whole-cell currents obtained in the presence of 10 mM Ba^{2+} . The mean r300 ratios (the fraction of the whole-cell current remaining at the end of a 300-ms pulse) are shown \pm SEM at four voltages from -10 to +20 mV for $\alpha 1E$ wt (black), $\alpha 1E$ R378K (light gray), $\alpha 1E$ R378A (dark gray), $\alpha 1E$ R378Q (white), $\alpha 1E$ R378G (gray), $\alpha 1E$ R378D (hatched), and $\alpha 1E$ R378E (cross-hatched) from left to right. At -10 mV, the r300 ratios for $\alpha 1E$ R378K and $\alpha 1E$ R378A were not significantly different from $\alpha 1E$ wt whereas those for $\alpha 1E$ R378Q were different at $p < 0.05$ and those for $\alpha 1E$ R378G, $\alpha 1E$ R378D, and $\alpha 1E$ R378E were significantly different at $p < 0.002$. The r300 ratios went from 0.04 ± 0.01 at -10 mV and 0.05 ± 0.02 at +20 mV ($n = 7$) for R378K, from 0.05 ± 0.01 at -10 mV to 0.06 ± 0.02 at +20 mV ($n = 4$) for R378A, from 0.13 ± 0.03 at -10 mV to 0.08 ± 0.02 at +20 mV ($n = 7$) for R378Q, from 0.27 ± 0.03 at -10 mV and 0.21 ± 0.01 at +20 mV ($n = 7$) for R378G, from 0.25 ± 0.03 at -10 mV to 0.19 ± 0.02 at +20 mV ($n = 14$) for R378D, and from 0.28 ± 0.03 at -10 mV to 0.23 ± 0.03 at +20 mV ($n = 14$) for R378E as compared with 0.05 ± 0.02 at -10 mV and 0.02 ± 0.01 at +20 mV ($n = 17$) for $\alpha 1E$. The number (n) of experiments is shown in parentheses. (B) Activation potentials were estimated from the mean normalized current-voltage relationships. The relative data points were plotted against the test voltage and were fitted to Boltzmann Eq. 2. The activation potentials were comparable for all mutants although they increased slightly from $\alpha 1E$ R378A \approx $\alpha 1E$ R378G \approx $\alpha 1E$ wt $<$ $\alpha 1E$ R378K \approx $\alpha 1E$ R378D \approx $\alpha 1E$ R378E $<$ $\alpha 1E$ R378Q. Table 1 contains the actual fit values.

and calcium-dependent inactivation mechanisms (Cens et al., 1999).

β -Subunit regulation is preserved in $\alpha 1E$ R378E and $\alpha 1C$ E462R channel mutants

As β -subunits are known to modulate the inactivation kinetics of the $\alpha 1$ -subunit and to bind to the AID site (Pragnell et al., 1994), the next series of experiments was under-

taken to examine the possibility that the altered inactivation kinetics of $\alpha 1E$ R378E and $\alpha 1C$ E462R were secondary to a modification in the coupling between $\beta 3$ and $\alpha 1$ -subunits as all previous experiments were performed in the presence of a full complement of auxiliary $\alpha 2b\delta$ - and $\beta 3$ -subunits. The objectives were twofold: 1) to explore whether modifications in nonconserved residues in AID could influence β -subunit modulation of the inactivation kinetics and 2) to evaluate whether the changes in the inactivation kinetics were intrinsically determined by the $\alpha 1$ -subunit. Mutant $\alpha 1E$ R378E and $\alpha 1C$ E462R were expressed in *Xenopus* oocytes in the presence ($\alpha 2b\delta/\beta 3$) and in the absence of $\beta 3$ with only $\alpha 2b\delta$ as ancillary subunit (Fig. 8). Whole-cell currents were recorded in the presence of 10 mM Ba^{2+} under strictly paired conditions (same day of expression, same oocyte batch) to minimize nonspecific effects. The presence of $\beta 3$ induced a leftward shift of the peak voltage by -10 mV for both $\alpha 1C$ E462R and $\alpha 1E$ R378E (results not shown), which is similar to what has been reported before for the wild-type channels (Parent et al., 1997). The voltage dependence of inactivation estimated from isochronal measurements at 5 s was shifted to the left in the presence of $\beta 3$ with $E_{0.5} = -11 \pm 2$ mV ($n = 4$) ($-\beta 3$) and $E_{0.5} = -26 \pm 2$ mV ($n = 6$) ($+\beta 3$) for $\alpha 1C$ E462R and $E_{0.5} = -26 \pm 1$ mV ($n = 3$) ($-\beta 3$) and $E_{0.5} = -44 \pm 1$ mV ($n = 3$) ($+\beta 3$) for $\alpha 1E$ R378E (results not shown). It thus appears that the mutants retained some modulation by the $\beta 3$ -subunit despite structural rearrangements in the main β -subunit-binding site.

The time courses of inactivation for $\alpha 1C$ E462R and $\alpha 1E$ R378E remained comparable to each other whether it was measured in the absence (left panel) or in the presence of $\beta 3$ (right panel) under all conditions except at -10 mV in the absence of $\beta 3$ as it can be inferred from the r300 ratio analysis (lower left panel). Moreover, the inactivation kinetics of both mutants remained significantly slower than $\alpha 1E$ ($p < 0.005$) under all conditions. The inactivation kinetics of $\alpha 1C/\alpha 2b\delta$ were not reported as its expression in oocytes never rose above background currents. Co-injection with the $\beta 3$ -subunit led to an apparent increase in inactivation kinetics for both mutants, which was confirmed by the r300 analysis. Hence, the inactivation kinetics remained similar for $\alpha 1E$ R378E and $\alpha 1C$ E462R in the absence of $\beta 3$.

DISCUSSION

Position R378 in the AID motif is critical in $\alpha 1E$ ($Ca_v2.3$) inactivation

In this study, the molecular determinants of voltage-dependent inactivation in the $\alpha 1E$ Ca^{2+} channel were investigated following mutations within the high-affinity β -subunit-binding site (AID) of the I-II linker. The AID motif is composed of a stretch of 18 amino acids located about at the 5' end of the I-II linker that reads QQXEXXLXGYX-

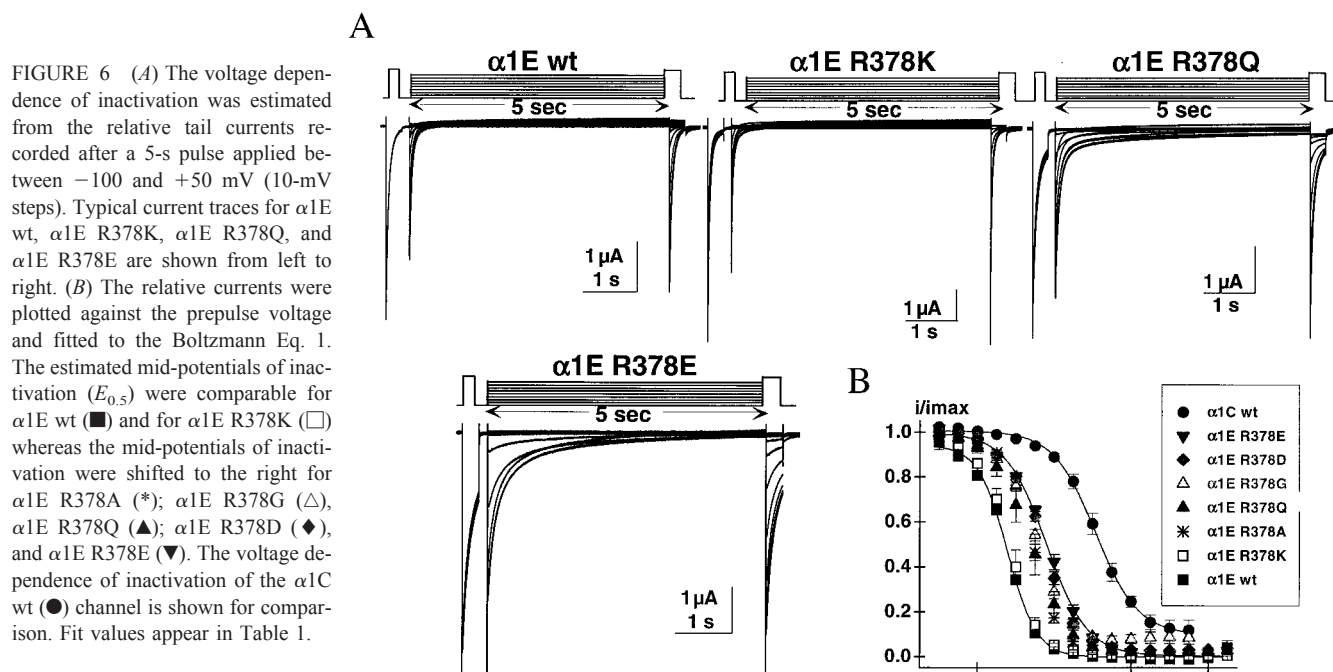


FIGURE 6 (A) The voltage dependence of inactivation was estimated from the relative tail currents recorded after a 5-s pulse applied between -100 and $+50$ mV (10-mV steps). Typical current traces for $\alpha 1E$ wt, $\alpha 1E$ R378K, $\alpha 1E$ R378Q, and $\alpha 1E$ R378E are shown from left to right. (B) The relative currents were plotted against the prepulse voltage and fitted to the Boltzmann Eq. 1. The estimated mid-potentials of inactivation ($E_{0.5}$) were comparable for $\alpha 1E$ wt (■) and for $\alpha 1E$ R378K (□) whereas the mid-potentials of inactivation were shifted to the right for $\alpha 1E$ R378A (*); $\alpha 1E$ R378G (△), $\alpha 1E$ R378Q (▲); $\alpha 1E$ R378D (◆), and $\alpha 1E$ R378E (▼). The voltage dependence of inactivation of the $\alpha 1C$ wt (●) channel is shown for comparison. Fit values appear in Table 1.

XWIXXXE. Considering that the I-II linker has recently emerged as an active participant in the inactivation of voltage-dependent calcium channels, we became interested in probing the role of the nonconserved residues in AID through a detailed mutational analysis at this site in $\alpha 1E$. Of the nine nonconserved residues, six positions involving significant changes in charge and/or size, as compared with the same sequence in $\alpha 1C$, were more thoroughly studied: R378E, N381K, R384L, A385D, D388T, K389Q, and K389E. All mutants tested expressed significant whole-cell currents after expression in *Xenopus* oocytes in the presence of $\alpha 2b\delta$ and $\beta 3$ -subunits. Some of these mutations were studied globally as they were included either in the triple (NRA-KLD), quadruple (NRAK-KLDQ), or the quintuple mutant (NRADK-KLDTQ). Neither multiple mutant displayed significant changes in their kinetics or voltage dependence of inactivation as compared with the wild-type $\alpha 1E$. In contrast, single point mutations of positively charged residues K to E at position K389 and the R to E mutation at position R378 significantly slowed the rate of inactivation. Of the two mutants, only the voltage dependence of inactivation of $\alpha 1E$ R378E was affected with $\sim +20$ -mV shift in its mid-potential of inactivation as compared with $\alpha 1E$ wt. A series of mutations at the R378 position indicated that the net charge carried by the side-chain could play a role in the inactivation kinetics, although the net charge carried by the residue could not explain by itself the effects of R378A, R378G, and R378Q (see below). Alterations in the inactivation properties occurred without any significant difference in the activation properties, hence suggesting that the inactivated state was intrinsically mod-

ified by the R378E mutation. The same observation was made for the family of mutants analyzed in this study. However this conclusion regarding the absence of change in the activation potential, remains preliminary in the absence of single-channel data. The relationship between activation and inactivation states cannot be simply overlooked as they are often coupled in voltage-dependent cation channels (Patil et al., 1998).

The reverse mutation E to R in $\alpha 1C$ was also shown to speed up inactivation and to shift the voltage dependence of inactivation to more negative membrane potentials (Fig. 7). The rates of $\alpha 1C$ E462R and $\alpha 1E$ R378E inactivation were both significantly altered and were almost indistinguishable at $+20$ mV. However, neither point mutation at this position in $\alpha 1E$ or $\alpha 1C$ channels could completely reverse the inactivation phenotype. Similarly, a series of experiments have shown that the I-II loop from either $\alpha 1B$ or $\alpha 1A$ produced intermediary inactivation kinetics when inserted into $\alpha 1E$ channels but could not completely reverse the inactivation kinetics of $\alpha 1E$ (Page et al., 1997). The role of the AID motif in inactivation kinetics has been previously demonstrated in $\alpha 1A$ and $\alpha 1C$ channels (Herlitze et al., 1997; Sokolov et al., 1999), but this is the first report that the nonconserved R (Arg) residue in the AID motif may contribute to the inactivation phenotype of $\alpha 1E$. Hence, the 5th position of the AID motif could be a universal determinant of inactivation in non-T-type Ca^{2+} channels.

The AID motif has been intensively studied in $\alpha 1A$, $\alpha 1B$, $\alpha 1C$, and $\alpha 1E$ channels mostly in terms of its role in G-protein modulation (Toth et al., 1996; Bourinet et al., 1996; Mehrke et al., 1997; Page et al., 1998). Interestingly,

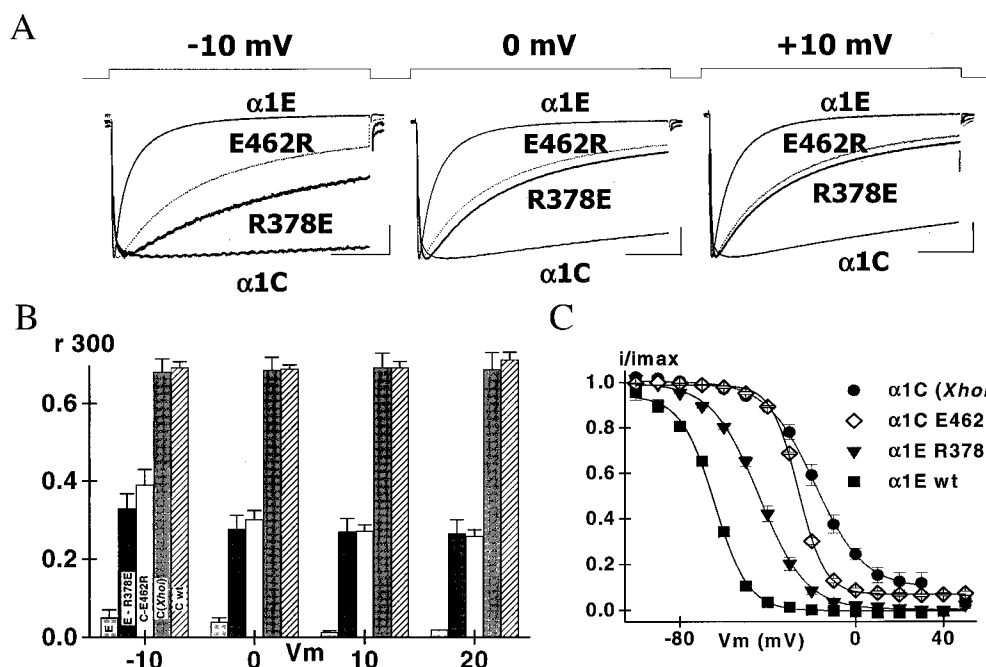


FIGURE 7 (A) Whole-cell currents obtained in the presence of 10 mM Ba^{2+} for $\alpha 1E$ wt, $\alpha 1C$ E462R, $\alpha 1E$ R378E, and $\alpha 1C$ wt were scaled and superimposed at the voltages of -10 (left), 0 (middle), and +10 mV (right). All $\alpha 1$ subunits were co-injected with $\alpha 2b\delta$ and $\beta 3$ subunits. Mutant $\alpha 1C$ E462R and $\alpha 1E$ R378E inactivated following a time course intermediary between $\alpha 1E$ and $\alpha 1C$ at all membrane potentials. (B) The mean r_{300} ratios calculated for the same channels are shown \pm SEM at four voltages from -10 to +20 mV for $\alpha 1E$ wt (light gray), $\alpha 1E$ R378E (dark gray), $\alpha 1E$ E462R (white), $\alpha 1C$ (XhoI) (gray), and $\alpha 1C$ wt (hatched) from left to right. The r_{300} ratios went from 0.05 ± 0.02 at -10 mV and 0.02 ± 0 at +20 mV ($n = 17$) for $\alpha 1E$, from 0.33 ± 0.04 at -10 mV to 0.27 ± 0.03 at +20 mV ($n = 14$) for $\alpha 1E$ R378E, from 0.39 ± 0.04 at -10 mV to 0.26 ± 0.02 at +20 mV ($n = 6$) for $\alpha 1C$ E462R, from 0.68 ± 0.03 at -10 mV to 0.69 ± 0.04 at +20 mV ($n = 6$) for $\alpha 1C$ (XhoI), and from 0.69 ± 0.01 at -10 mV to 0.71 ± 0.02 at +20 mV ($n = 9$) for $\alpha 1C$ wt. (C) The voltage dependence of inactivation was estimated from the relative tail currents recorded after 5-s pulses. Normalized currents were fitted to the Boltzmann Eq. 1. The estimated mid-potential of inactivation ($E_{0.5}$) for $\alpha 1C$ E462R (◇) was more negative than for the $\alpha 1C$ (XhoI) construct (●) but more positive than for $\alpha 1E$ R378E (▼) and $\alpha 1E$ wt (■). The fit values are shown in detail in Table 1.

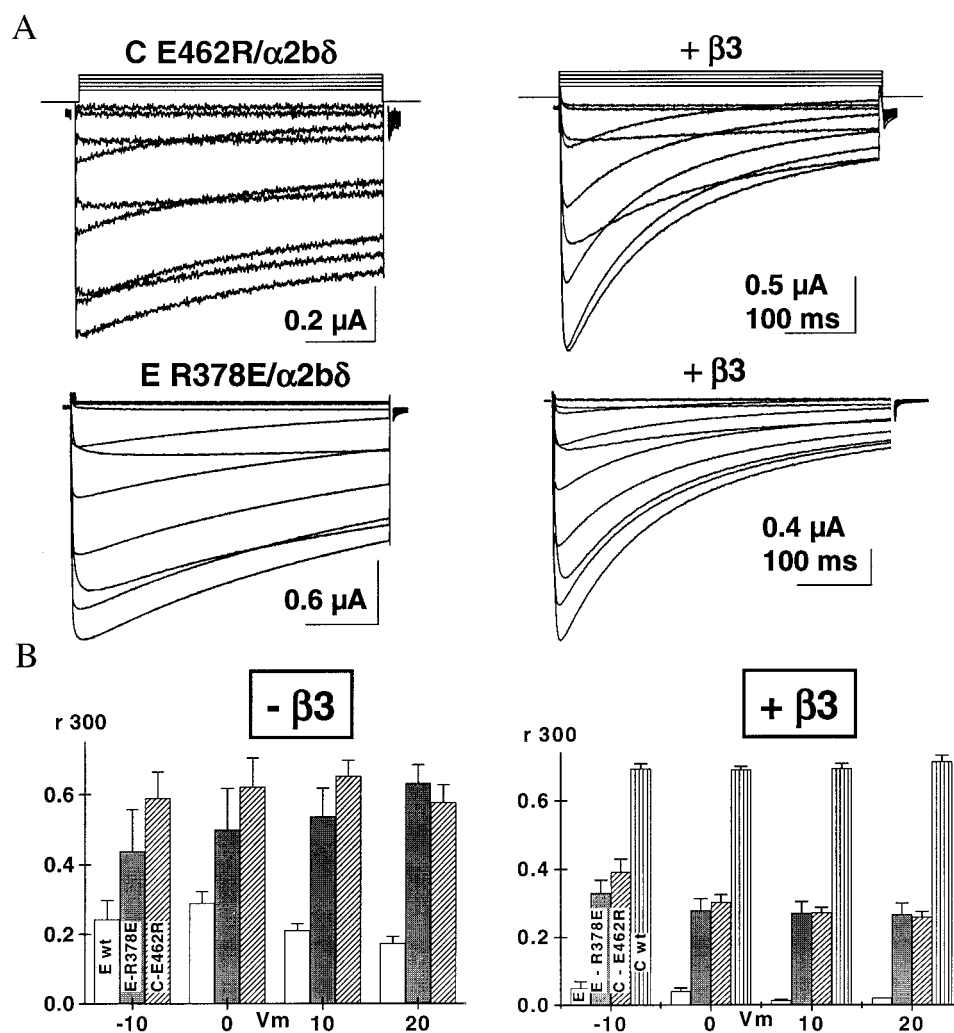
the R-to-E mutation at the same position of the AID motif has also been shown to eliminate G-protein regulation in $\alpha 1A$ (Herlitz et al., 1997). However, it failed to affect G-protein modulation in $\alpha 1B$ channels (Zhang et al., 1996) as did the reverse mutation E to R fail to bestow G-protein modulation to $\alpha 1C$ channels (Zhang et al., 1996). Despite these small discrepancies, these results suggest that the I-II linker may be specialized to serve as a modulatory domain for Ca^{2+} channel gating with the Arg (R) at position 5 playing a determinant role in many overlapping processes (Zamponi et al., 1997; DeWaard et al., 1997; Herlitz et al., 1997).

A positive residue in the QQXXER motif triggers fast inactivation of $\alpha 1E$ ($Ca_v2.3$)

Only the mutation of the positively charged residue (R378) to negatively charged ones (E or D) at the 5th position of the QQXXER motif affected both the kinetics and voltage dependence of inactivation. Experiments were undertaken to investigate the role of electrostatic interaction in the inactivation kinetics of $\alpha 1E$ with additional mutants R378K (positive), R378A (nonpolar and neutral), R378G (polar but

neutral), R378Q (polar but neutral), and R378D (negative). At first glance, an electrostatic interaction appears to play a determinant role in the inactivation properties, as $\alpha 1E$ R378K was the only mutant to reproduce the wild-type inactivation kinetics and voltage dependence. In contrast, negatively charged mutants R378D and R378E showed the slowest inactivation properties. However, such an interpretation falls short of explaining the behavior of neutral mutants R378A, R378G, and R378Q. Mutant R378A inactivated like R378K between -10 mV and +20 mV. R378Q displayed intermediary inactivation kinetics at -10 mV but tended to inactivate like R378K at +20 mV. R378G produced, on the other hand, slow inactivation kinetics comparable to R378D and R378E at all membrane potentials. Hence, there was no simple correlation between the inactivation kinetics of R378 mutants (R378E, R378D, R378K, R378A, R378Q, and R378G) and any single physicochemical property (charge, polarity, hydrophobicity, and hydrophilicity). The intermediary behavior of R378Q could be partly explained by the relative polarity of its side-chain as compared with R378A. However, R378G, which also bears a neutral but slightly polar residue at the same position, diverged from that prediction. Glycine residues are known

FIGURE 8 β 3-Subunit modulation of mutants α 1E R378E and α 1C E462R. (A) The α 1E R378E and α 1C E462R mutants were expressed in *Xenopus* oocytes in the presence of α 2b δ (left) and α 2b δ / β 3 subunits (right). Whole-cell currents were recorded with 10 mM Ba^{2+} using a series of pulses from -40 mV to $+60$ mV from a holding potential of -80 mV. Whole-cell currents peaked at $+10 \pm 1$ mV ($n = 4$) for α 1C E462R/ α 2b δ and $+1 \pm 1$ mV ($n = 7$) for α 1C E462R/ α 2b δ / β 3, at $+10 \pm 1$ mV ($n = 3$) for α 1E R378E/ α 2b δ , and at 0 ± 0.2 mV ($n = 3$) for α 1E R378E/ α 2b δ / β 3. In both cases, co-injection with the β 3-subunit led to a significant increase in their respective inactivation kinetics. Time scale is 100 ms throughout. (B) At $+10$ mV, r_{300} went from 0.21 ± 0.02 for α 1E/ α 2b δ ($n = 3$) to 0.01 ± 0.005 for α 1E/ α 2b δ / β 3 ($n = 6$), from 0.54 ± 0.08 for α 1E R378E/ α 2b δ ($n = 3$) to 0.27 ± 0.03 for α 1E R378E/ α 2b δ / β 3 ($n = 9$), and from 0.65 ± 0.05 for α 1C E462R/ α 2b δ ($n = 4$) to 0.27 ± 0.02 for α 1C E462R/ α 2b δ / β 3 ($n = 6$).



to confer flexibility to polypeptides (Creighton, 1993), which could considerably lessen whatever interaction takes place at this position. Although sometimes classified with alanine in terms of its physicochemical properties, glycine mutants have been shown to behave distinctively from alanine mutants when introduced into membrane proteins. For instance, in an extensive mutagenesis study of the α 1-subunit of the human glycine receptor, Gly mutants at position 288 were found to display EC_{50} similar to the Glu mutants and significantly lower than the wild-type channel with an Ala residue at this position (Yamakura et al., 1999).

Mutations at the fifth position of the AID motif did not prevent β -subunit modulation

The AID motif appears to be cumulating critical determinants for Ca^{2+} channel gating (Herlitze et al., 1997; Dolphin, 1998), namely, in the context of this work (Walker and DeWaard, 1998), voltage-dependent inactivation and β -subunit modulation. Although it had been previously reported that the QXXER motif could play a role in voltage-depen-

dent inactivation, few groups have addressed the possibility that a modification in β -subunit modulation could explain the altered inactivation properties as the mutation involved a residue considered noncritical for β -subunit binding. By investigating the inactivation properties of α 1E R378E and α 1C E462R in the presence and in the absence of β 3, we showed that the R-to-E mutation at the nonconserved position 5 of the AID motif failed to prevent β -subunit modulation. Both mutants displayed the typical hallmarks of β -subunit modulation in Ca^{2+} channels, namely, the increase in peak currents, negative shifts in the voltage dependence of activation and inactivation, and increases in the inactivation kinetics (Fig. 8). These data hence confirmed that the changes in the inactivation kinetics observed in α 1E R378E and α 1C E462R were intrinsically determined by changes in the α 1-subunit. We, however, cannot completely rule out the possibility that mutations within the AID motif could somewhat modify the protein-protein interaction between the mutated α 1- and the β -subunits, for modulation and protein interaction could be distinct processes. It is thus possible that β -subunits could modulate the α 1-subunit

independently of their ability to bind to the main AID (Yamaguchi et al., 1998). This distinction appears confirmed by a recent study that showed that mutations of the conserved Y (Tyr) residue in $\alpha 1C$ (Y467S) had no significant effect on β -subunit-induced modulation of whole-cell currents (Gerster et al., 1999).

Could a hinged-lid mechanism explain voltage-dependent inactivation in Ca^{2+} channels?

It is increasingly suggested that the I-II linker of HVA $\alpha 1$ -subunits could behave as an inactivating blocking particle in channels (Bourinet et al., 1999; Cens et al., 1999; Stotz et al., 2000) by analogy to the hinged-lid mechanism described in Na^+ channels. In this scheme, there could be a role for R378 contributing to a conformational change that could eventually cause occlusion of the channel pore. The proposition that inactivation in $\alpha 1E$ channels involves the I-II linker as the inactivation gate remains, however, premature at this point in the absence of a three-dimensional structure.

Our data support a major contribution of R378 to the inactivation properties (kinetics and voltage dependence) of $\alpha 1E$. The mutation of a neighboring positive residue in K389E slowed the inactivation kinetics but failed to significantly influence the voltage dependence of inactivation. It remains to be seen, however, whether mutations at this site reverberate on other sites, critical for inactivation, that were not studied in this work. Indeed, mutations at the R378 position failed to completely eliminate voltage-dependent inactivation, indicating that other loci are required to fully account for voltage-dependent inactivation in $\alpha 1E$ channels. Some other contributing sites could include, but are not restricted to, residues located downstream to the AID motif or residues located in the C-terminus. Indeed, splice variants in the I-II linker produced $\alpha 1A$ channels with altered inactivation phenotypes (Bourinet et al., 1999), and mutations in the C-terminus were shown to slow voltage-dependent inactivation (Bernatchez et al., 1998). By analogy with inactivation in Na^+ and K^+ channels, it is highly possible that sites responsible for voltage-dependent inactivation in the $\alpha 1E$ Ca^{2+} channel are distributed along its primary structure.

Our data on the molecular determinants of inactivation in $\alpha 1E$ Ca^{2+} channel inactivation highlight some critical differences with the mechanism of fast inactivation in brain Na^+ channels. Point mutations in the I-II linker failed to affect the voltage dependence of activation and inactivation of brain Na^+ channels (Li et al., 1992). Furthermore, charge neutralization in the III-IV linker of the brain Na^+ channel was not found to affect its fast inactivation kinetics (Patton et al., 1992). The locus of inactivation in brain Na^+ channels is composed of three hydrophobic residues, the IFM motif (West et al., 1992). In an extensive site-directed mutagenesis of the Phe (F) site, Catterall and colleagues have shown that normal inactivation kinetics in Na^+ chan-

nels requires hydrophobic residues (Kellenberger et al., 1997). In other words, amino acids with aliphatic and aromatic side-chains stabilized the interaction with the putative inactivation receptor activated state whereas hydrophilic ones tended to disrupt it (Kellenberger et al., 1997). Our data suggest that the inactivated state in $\alpha 1E$ Ca^{2+} channels could be disrupted by the presence of a negatively charged residue at position R378. Moreover, positively charged residues at position R378 are required to yield the fastest inactivation kinetics whereas nonpolar residues could mimic to a certain extent the normal inactivation kinetics. As the residues in the inactivation ball in the N-terminus of the *Shaker* K^+ channels were mostly hydrophobic (Hoshi et al., 1991), it appears that the molecular mechanisms explaining inactivation in $\alpha 1E$ channels could differ substantially from the ones in Na^+ and K^+ channels, especially regarding the nature of the interaction at the inactivation site. It is, in addition, too early to draw any parallel between the observation that inactivation in $\alpha 1A$, $\alpha 1B$, and $\alpha 1E$ Ca^{2+} channels could occur from intermediate closed states (Patil et al., 1998) and the general description of voltage-dependent inactivation in many voltage-gated sodium (Armstrong and Bezanilla, 1977; Bean, 1981; Kuo and Bean, 1994) and potassium channels (Hoshi et al., 1990; Demo and Yellen, 1991) where inactivation rates accelerate sharply and progressively in proximity to the open state.

The ongoing debate regarding the molecular locus of voltage-induced inactivation in Ca^{2+} channels underscores the intricate and complex nature of this mechanism. Recent data gathered from mutagenesis studies appear to converge toward residues within the I-II linker in $\alpha 1E$ (Stotz et al., 2000; our data), $\alpha 1C$ (Herlitze et al., 1997; our data), and $\alpha 1A$ channels (Ellinor et al., 1993; Herlitze et al., 1997; Bourinet et al., 1999). As the I-II linker of T-type $\alpha 1G$, $\alpha 1H$, and $\alpha 1I$ does not display a high degree of homology with other $\alpha 1$ -subunits, transitions to the inactivated state in these channels probably involve other molecular determinants.

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