Intracellular Ca^{2+} Inactivates L-Type Ca^{2+} Channels with a Hill Coefficient of ~1 and an Inhibition Constant of ~4 μM by Reducing Channel's Open Probability

G. F. Höfer,* K. Hohenthanner,* W. Baumgartner,* K. Groschner,# N. Klugbauer,§ F. Hofmann,§ and C. Romanin*

*Institute for Biophysics, University of Linz, A-4040 Linz, Austria, #Institute for Pharmacology and Toxicology, University of Graz, A-8010 Graz, Austria, and §Institute for Pharmacology and Toxicology, Technical University Munich, D-80802 München, Germany

ABSTRACT The patch-clamp technique was used to characterize the mechanism of Ca^{2+}-induced inactivation of cardiac L-type Ca^{2+} channel $\alpha_{1C\text{-}a}$ + β_3 subunits stably expressed in CHO cells. Single Ca^{2+} channel activity was monitored with 96 mM Ba^{2+} as charge carrier in the presence of 2.5 μM (−)BAYK 8644 and calpastatin plus ATP. This enabled stabilization of channel activity in the inside-out patch and allowed for application of steady-state Ca^{2+} concentrations to the intracellular face of excised membrane patches in an attempt to provoke Ca^{2+}-induced inactivation. Inactivation was found to occur specifically with Ca^{2+} since it was not observed upon application of Ba^{2+}. Ca^{2+}-dependent inhibition of mean Ca^{2+} channel activity was characterized by a Hill coefficient close to 1. Ca^{2+} binding to open and closed states of the channel obtained during depolarization apparently occurred with similar affinity yielding half-maximal inhibition of Ca^{2+} channel activity at ~4 μM. This inhibition manifested predominantly in a reduction of the channel's open probability whereas availability remained almost unchanged. The reduction in open probability was achieved by an increase in first latencies and a decrease in channel opening frequency as well as channel open times. At high (12–28 μM) Ca^{2+} concentrations, 72% of inhibition occurred due to a stabilization of the closed state and the remaining 28% by a destabilization of the open state. Our results suggest that binding of one calcium ion to a regulatory domain induces a complex alteration in the kinetic properties of the Ca^{2+} channel and support the idea of a single EF hand motif as the relevant Ca^{2+} binding site on the α_1 subunit.

INTRODUCTION

Ca^{2+} entry through voltage-activated Ca^{2+} channels plays a crucial role in the transmembrane signaling of cellular processes such as muscle contraction and neurotransmitter secretion (Eckert and Chad, 1984; Carbone and Swandulla, 1989; McDonald et al., 1994). Elevation of intracellular Ca^{2+} due to opening of L-type Ca^{2+} channels inhibits Ca^{2+} entry. This process, termed Ca^{2+}-induced inactivation, serves as an important, negative feedback mechanism by speeding Ca^{2+} channel inactivation.

As molecular mechanisms initiating inactivation direct binding of Ca^{2+} to the channel or to an associated regulatory protein (Sherman et al., 1990) as well as Ca^{2+}-mediated dephosphorylation (Chad and Eckert, 1986; Armstrong, 1989) have been proposed. Recent studies favor direct binding of Ca^{2+} to a cytoplasmic domain (Yue et al., 1990; Imredy and Yue, 1992, 1994; Haack and Rosenberg, 1994). L-type Ca^{2+} channels are constituted of five subunits, α_1, α_2, β, γ, and δ, of which the α_1 is as yet best characterized in terms of structure-function relationships (DeFelice, 1993; Hofmann et al., 1994; Isom et al., 1994; Varadi et al., 1995). Expression of the cardiac α_1 subunit alone has been found sufficient for channel activity with apparent Ca^{2+}-sensitive inactivation (Neely et al., 1994; Zong and Hofmann, 1996). An EF hand motif characteristic for Ca^{2+} binding in proteins has been localized in the carboxyl tail close to the IVS6 of the α_1 subunit of the Ca^{2+} channel (Babitch, 1990). Recently, molecular evidence has been presented (DeLeon et al., 1995) suggesting that the EF hand Ca^{2+} binding motif of the cardiac α_{1C} subunit provides the Ca^{2+} binding site that is responsible for Ca^{2+}-sensitive inactivation. This has been elegantly demonstrated, in particular, by a chimeric α_1 subunit in which 51 amino acids containing the EF hand motif of the cardiac $\alpha_{1C\text{-}a}$ subunit have been substituted by the corresponding sequence of the brain α_{1E} subunit, which lacks Ca^{2+}-induced inactivation (DeLeon et al., 1995). As a consequence, the resultant chimera has lost Ca^{2+}-sensitive inactivation. A lower or even lacking Ca^{2+} affinity of the conferred EF hand motif might be one possible explanation for this finding. Thus, determination of the inhibition constant (K_i) for Ca^{2+} channel inactivation by Ca^{2+} would significantly contribute to the molecular understanding of the role of EF hand motifs in mediating inactivation.

Usually, Ca^{2+}-sensitive inactivation is demonstrated by comparison of the inactivation observed with Ca^{2+} versus Ba^{2+} as charge carrier. This experimental design, which measures channel inactivation as a direct consequence of Ca^{2+} permeation, renders determination of the K_i of Ca^{2+}-induced inactivation rather impracticable. This would instead require application of steady-state Ca^{2+} concentrations to the inner face of Ca^{2+} channels.

Received for publication 30 September 1996 and in final form 8 July 1997.

Address reprint requests to C. Romanin, Institute for Biophysics, University of Linz, Altenbergerstrasse 69, A-4040 Linz, Austria. Tel.: ++43-732-2468-9272; Fax: ++43-732-2468-10; E-Mail: christoph.romanin@jk.uni-linz.ac.at.

0006-3495/97/10/1857/09 $2.00

Therefore, this study used an alternative experimental approach previously established with native Ca^{2+} channels (Romanin et al., 1992) to characterize in detail the effect of intracellular Ca^{2+} on expressed cardiac Ca^{2+} channel $\alpha_{1C\text{-}a}$ + β_3 subunits in CHO cells. Specifically, single Ca^{2+} channel activity was monitored with Ba^{2+} as charge carrier enabling application of defined Ca^{2+} concentrations to the cytoplasmic side of an inside-out patch containing functionally active Ca^{2+} channels. Our results suggest a 1:1 interaction of Ca^{2+} and the Ca^{2+} channel with a K_i of ~4 μM leading to channel inactivation by a reduction in open probability rather than availability.

MATERIALS AND METHODS

Materials

Calpastatin (P-0787) was purchased from Sigma (Vienna, Austria) and was dialyzed overnight versus bath solution (see below). HAM's F12 medium, penicillin/streptomycin, and histidinol were obtained from Gibco BRL (Vienna, Austria). Geneticin (G-418) was from Sigma (Vienna, Austria).

Cell preparation, transfection, and culture

Guinea-pig ventricular myocytes were prepared as described (Romanin et al., 1991) and were used for electrophysiological measurements within 12 h. The construction and selection of the CHO$C\alpha_1\beta_3$ cell line (clone 8), which contains the entire protein coding region of the rabbit cardiac $\alpha_{1C\text{-}a}$ subunit (Biel et al., 1991) together with the coding region of the β_3 subunit (Hullin et al., 1992) have already been described (Welling et al., 1993; Lacinova et al., 1995). CHO cells were cultured in HAM's F12 supplemented with 10% FCS, penicillin/streptomycin, 0.48 g/l histidinol, and 0.178 g/l geneticin in a humidified atmosphere at 37°C and 5% CO_2.

Electrophysiological measurements

Single channel patch-clamp recordings (Hamil et al., 1981) were obtained from guinea-pig ventricular myocytes or CHO$C\alpha_1\beta_3$ cells using a List L/M EPC 7 amplifier. Borosilicate glass patch pipettes had resistances between 3.8 and 6.3 MΩ after filling with a solution containing (in mM): 0.0025 (−)-BAYK 8644, 96 $BaCl_2$, 5 Hepes/Na, pH 7.35. Bath solution consisted of (in mM): 110 aspartic acid, 20 KCl, 2 EGTA, 2 $MgCl_2$, 20 Hepes/K, pH 7.4. L-type Ca^{2+} channel activity was stabilized in the inside-out patch (Romanin et al., 1991; Seydl et al., 1995) using Sigma (2 U/ml) calpastatin and ATP/Na_2 (1 mM), which both were usually perfused into the bath chamber before patch excision. Inclusion of BAYK 8644 in pipette solution enabled persistent Ca^{2+} channel stabilization for at least 30 min. In the absence of BAYK 8644, stabilization of Ca^{2+} channel activity by calpastatin is much less efficient (Romanin et al., 1991) and experiments of such kind are extremely difficult to obtain. Single channel activities were recorded first in the cell-attached (c.a.) patch, and then after patch excision in the inside-out (i.o.) configuration during repetitive (0.25 Hz) depolarizations for 1.45 s to 0 mV. The holding potential was −45 mV and −80 mV in experiments with ventricular myocytes and CHO cells, respectively. The holding potential of −45 mV in ventricular myocytes was used to prevent T-type Ca^{2+} channel activation and is not expected to significantly alter single channel activity compared to −80 mV (Cavalie et al., 1986). Single channel traces were filtered at 0.5 kHz (−3 dB 2-pole Bessel filter) and digitized at 1.5 kHz. Alternatively, a fast-pulse protocol was employed in experiments used for accurate determination of single channel characteristics (see below) with repetitive depolarizations (0.66 Hz) applied for 0.475 s from a holding potential of −80 mV to 0 mV. These single channel traces were filtered at 1 kHz and digitized at 4 kHz. With both types of pulse protocols Ca^{2+}-dependent reduction of mean channel activity ($N \cdot p$) exhibited a similar concentration dependence.

Analysis of electrophysiological data

Due to the paucity of experiments with one single channel, evaluation of the effect of Ca^{2+} on Ca^{2+} channel activity was primarily based on the time course of mean channel activity $N \cdot p$ (Seydl et al., 1995) determined for each depolarizing voltage pulse (sweep). Single channel sweeps were idealized using the 50% threshold method. The mean current during depolarization (I) was determined and divided by the unitary current amplitude (i) to yield $N \cdot p$. Ca^{2+}-induced inactivation of channel activity was further judged by an alteration of the maximum number of channels present in the patch (N), their channel availability (P_s), and open probability (p_o) employing a recently developed analysis method (Schmid et al., 1995). P_s was defined by the ratio (× 100 in percent) of depolarizing sweeps with channel activity to the total number of sweeps, and p_o represents a channel's open probability in non-blank sweeps. As ensemble average currents in the presence of 5 μM Ca^{2+} show moderate (~50%) inactivation during 475-ms depolarizations, simulations were performed to carefully test for applicability of our analytical methods, which ideally would require steady state. According to a kinetic scheme as published for the L-type Ca^{2+} channel (Imredy and Yue, 1994), kinetic constants and inactivation behavior were slightly modified to match experimental single channel characteristics at 5 μM Ca^{2+} leading to ensemble average currents with an inactivation of ~70%.

$$C \underset{0.28}{\overset{0.21}{\rightleftharpoons}} C \underset{1.2}{\overset{0.36\ ms^{-1}}{\rightleftharpoons}} O$$

It has been assumed that the inactive state can be reached from each state in the scheme by the same rate (0.004 ms^{-1}) yielding ~70% macroscopic inactivation. The results presented in Table 1 revealed only minor errors (<2%) in determination of p_o and P_S, and ~5% error in the calculation of mean first latency, indicating applicability of our analytical methods. Furthermore, a new procedure was developed for calculation of the number of available channels in each sweep (Baumgartner et al., 1997) in order to allow for estimation of single channel parameters such as p_o and first latency on a sweep to sweep basis. This was performed by calculation of the posterior probability of the number of channels per sweep using a Bayesian estimator. The posterior probability was used for calculation of first latency distributions employing a maximum likelihood estimator (Baumgartner et al., 1997). Alternatively, empirical distributions of first latencies were obtained by multiplying the time to first opening by the most likely number of channels for each sweep. This procedure, based on the analysis of the number of functional channels in individual sweeps, corrects for the effect of a limited Ca^{2+} channel availability and yields a good approximation in the case of two time constants as found with simulated data (Baumgartner et al., 1997). As first latencies are long compared to the sweep length, particularly in the presence of high Ca^{2+}, τ_1, and τ_2 as well as their respective proportions, were calculated as described for two-sided censored data (Colquhoun and Sigworth, 1995). Mean first latencies calculated for a finite sweep length under control conditions and in the presence of high (12–28 μM) Ca^{2+} were used for determination of the

TABLE 1 Programmed and calculated parameters of single channel simulations yielding ensemble average currents with ~70% inactivation

Parameter	Programmed	Calculated	Relative error (%)
p_o	0.0493	0.0496	0.8
P_S	0.627	0.637	1.6
c_1	0.60	0.57	5.0
τ_1	30 ms	31 ms	3.3
τ_2	150 ms	132 ms	12.0
τ_{mean}	78.0 ms	74.4 ms	5.0

relative reduction in p_o due to the Ca^{2+}-induced increase in first latency. Mean first latencies calculated for a finite sweep length were also used for correction of the opening frequency in order to separate the effect of Ca^{2+} on first latency from that on the opening frequency. The opening frequency was calculated by $NU/(NS \cdot N \cdot P_S \cdot SL)$, where NU, NS, N, P_S, and SL refer to the number of upward deflections, total number of sweeps, total number of channels, availability, and sweep length (475 ms), respectively. A correction of opening frequency for first latency was performed by dividing it through the effective sweep length obtained by 475 ms minus mean first latency. Open times were determined from non-overlapping channel openings. Since p_o was usually <10% and $N \leq 3$, open time distributions are expected not to be biased. Distributions were corrected for missed events by excluding events smaller than 1 ms.

Determination of free Ca^{2+} concentrations

Total Ca^{2+} and Ba^{2+} concentrations required for desired free divalent cation concentrations were calculated (Fabiato, 1988) employing the apparent stability constants for Ca^{2+}-EGTA (1.244×10^7) and Ba^{2+}-EGTA ($3.545 \times 10^{4)}$ at pH = 7.35 and 22°C. In addition, Ca^{2+} concentrations as indicated here were directly measured by a calcium macroelectrode as described (Romanin et al., 1992). The free Ca^{2+} concentration of bath solution containing calpastatin and ATP without any additions of Ca^{2+} was estimated as <20 nM (Romanin et al., 1992). Addition of Ba^{2+} to bath solution yielding 28 μM free Ba^{2+} concentration was estimated not to increase free Ca^{2+} concentration above 20 nM.

RESULTS

Ca^{2+} channel activity of expressed α_1 and β_3 subunits is sensitive to cytoplasmic Ca^{2+} but not to Ba^{2+}

Mean Ca^{2+} channel activity ($N \cdot p$) of $\alpha_{1C\text{-}a}$ and β_3 subunits stably expressed in CHO cells exhibited a run-down upon patch excision (Fig. 1) similar to that of native cardiac Ca^{2+} channels (Romanin et al., 1991; Seydl et al., 1995). Application of calpastatin and ATP to the intracellular side of the membrane patch resulted in almost complete recovery of Ca^{2+} channel activity, which remained stable for at least 30 min. This finding enabled us to examine for the effect of intracellular Ca^{2+} in an attempt to provoke Ca^{2+}-induced inactivation of Ca^{2+} channel activity. A typical experiment is shown in Fig. 2. Elevation of the Ca^{2+} concentration at the intracellular side of the membrane from <20 nM to 23 μM Ca^{2+} induced a dramatic decrease in Ca^{2+} channel activity, which recovered upon wash-out of Ca^{2+}. The decrease was a specific effect of Ca^{2+} in this concentration range, since Ca^{2+} channel activity remained unchanged upon elevation of the intracellular Ba^{2+} concentration to 28 μM, as shown in Fig. 3. This result demonstrated that the observed inhibition exhibited a typical feature of the Ca^{2+}-induced inactivation process and encouraged us to examine this inactivation in more detail.

Ca^{2+} channel inactivation is characterized by a 1:1 interaction of Ca^{2+} with a binding site of an affinity of ~4 μM

Ca^{2+}-induced inactivation of Ca^{2+} channel activity was dependent on the concentration of intracellular Ca^{2+} as

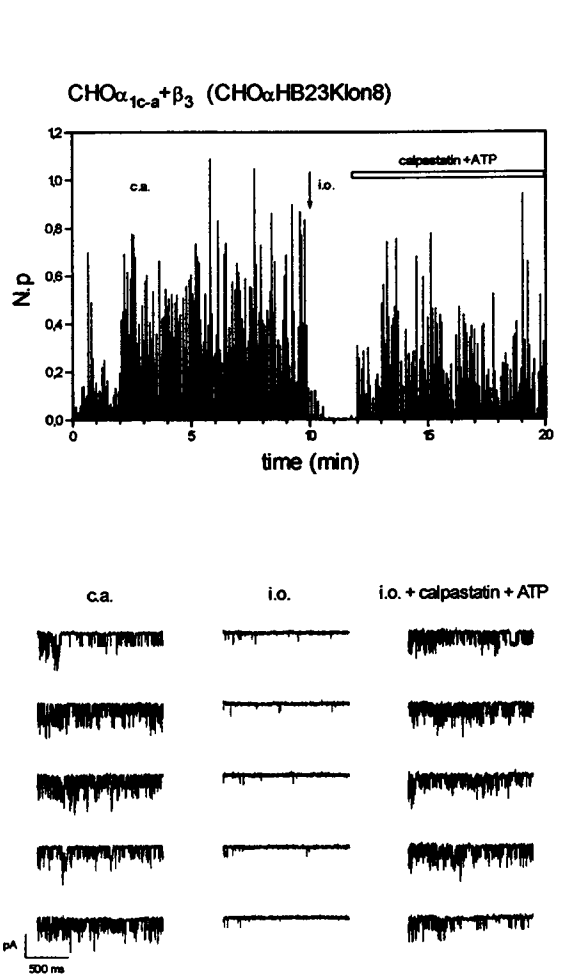

FIGURE 1 Run-down of Ca^{2+} channel activity of $\alpha_{1C\text{-}a}$ + β_3 subunits expressed in CHO cells is reversed by calpastatin + ATP. (*A*) Diary plot of mean channel activity ($N \cdot p$) in the cell-attached (c.a.) patch and following patch excision in the inside-out (i.o.) patch. Application of calpastatin + ATP is indicated. (*B*) Corresponding single channel traces in the c.a. configuration and in the i.o. patch before and after addition of calpastatin + ATP.

depicted in Fig. 4. In the examined range of 1–22 μM Ca^{2+}, the K_i for Ca^{2+}-induced inactivation of expressed Ca^{2+} channels was estimated as 4.4 μM (*solid line*) which was similar to that of native cardiac Ca^{2+} channels (K_i = 3.0 μM). A Hill plot analysis revealed a Hill coefficient close to 1 as depicted in the inset in Fig. 4, which suggests a 1:1 molecular interaction of Ca^{2+} with a site mediating inactivation, both in expressed and native Ca^{2+} channels. In order to allow for a mechanistic interpretation as to how intracellular Ca^{2+} reduces channel activity, we examined next whether Ca^{2+} affected the total number of channels (N), their availability to open (P_s) or/and the open probability of available channels (p_o). Since almost all experiments were performed with multi-channel patches, estimation of the above channel parameters was carried out by a recently established analysis method based on a maximum likelihood estimator (Schmid et al., 1995).

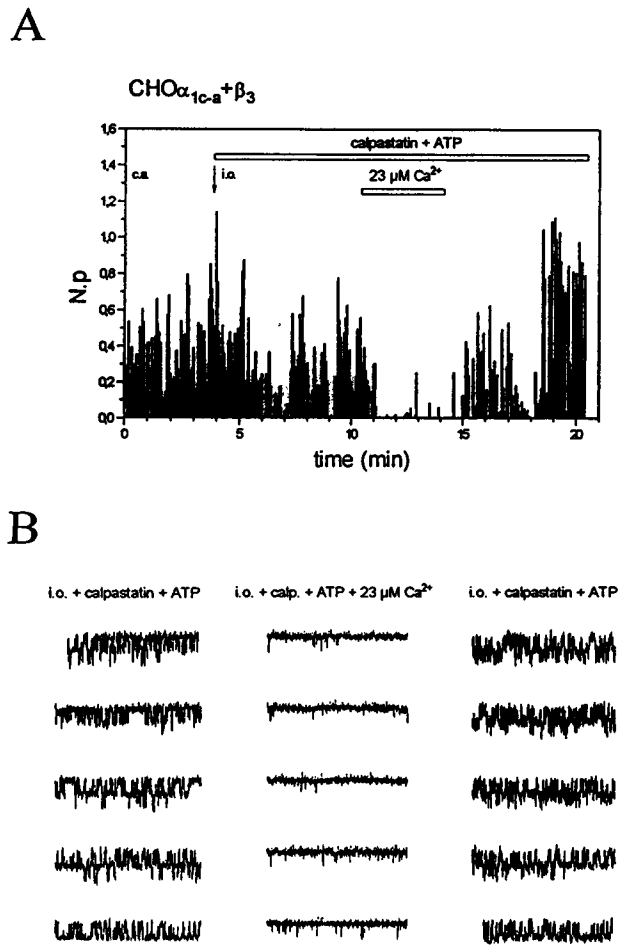

FIGURE 2 Intracellular Ca^{2+} induces a reversible inactivation of Ca^{2+} channel activity of expressed $\alpha_{1C\text{-}a}$ + β_3 subunits. (*A*) Diary plot of mean Ca^{2+} channel activity ($N \cdot p$) in the c.a. and i.o. patch. Application of calpastatin + ATP as well as 23 μM Ca^{2+} is indicated. (*B*) Corresponding single channel traces recorded in the i.o. patch show a prominent decrease in channel activity following addition of 23 μM Ca^{2+}. After its removal, channel activity completely recovered.

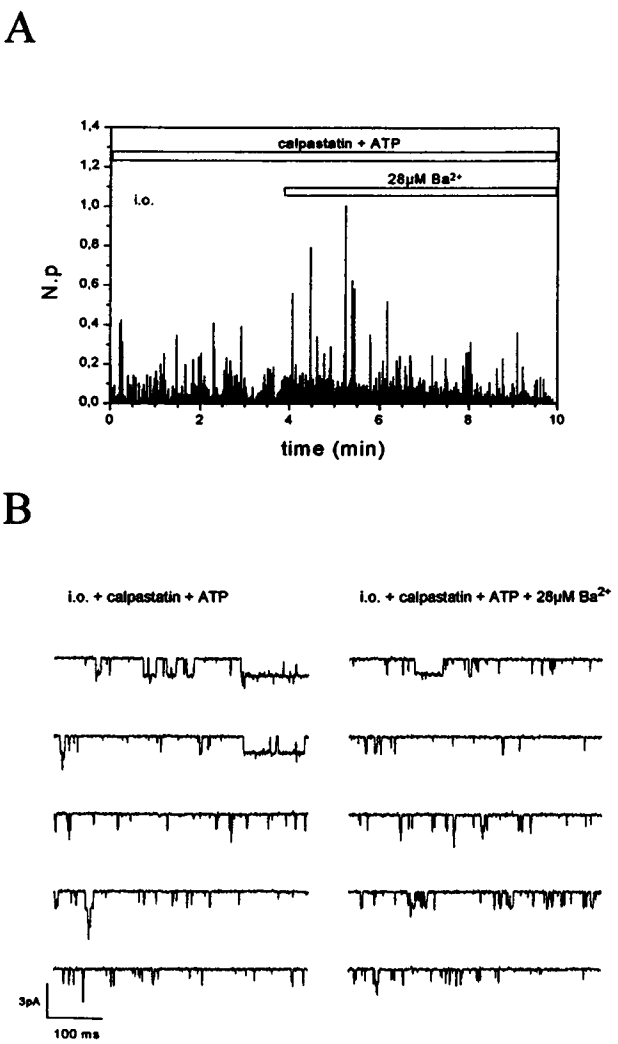

FIGURE 3 Intracellular Ba^{2+} fails to induce inactivation of Ca^{2+} channel activity of expressed $\alpha_{1C\text{-}a}$ + β_3 subunits. (*A*) Diary plot of mean Ca^{2+} channel activity ($N \cdot p$) in the i.o. patch stabilized by calpastatin + ATP. Application of Ba^{2+} is indicated. (*B*) Corresponding single channel traces evidencing a lack of effect of 28 μM Ba^{2+} on Ca^{2+} channel activity.

Ca^{2+}-induced inactivation is caused by a reduction in p_o rather than P_s

While N was not affected by intracellular Ca^{2+}, Fig. 5 shows the dependence of P_s and p_o on increasing Ca^{2+} concentrations (Fig. 5 *A, filled symbols, solid lines*) together with single channel traces and their corresponding ensemble average currents at three Ca^{2+} concentrations (Fig. 5 *B*, 1, 5, and 12 μM Ca^{2+}). Under control conditions in the inside-out patch (<20 nM Ca^{2+}), Ca^{2+} channel P_s and p_o were determined as 62 ± 9% and 9 ± 6% (n = 16, mean ± SD), respectively. Upon increasing Ca^{2+} within the concentration range from 1 to 28 μM, Ca^{2+} channel p_o was strongly reduced, while P_s was moderately affected. An effect on P_s would be overestimated, if channel first latency is longer than the actual sweep length. A significant increase in channel first latency has been indeed reported for native Ca^{2+} channels subjected to Ca^{2+}-induced inactivation (Imredy and Yue, 1994). To take this into account we developed a new method required for analysis of multi-channel data yielding an estimation of the number of available channels in each sweep (Baumgartner et al., 1997; see Materials and Methods). This enabled us to calculate p_o and first latency for each sweep as shown in Fig. 6. Diary plots from a typical experiment demonstrating the effect of 12 μM Ca^{2+} on $N \cdot p$, p_o, and first latency (Fig. 6, *A–C*, respectively) revealed that p_o determined for each sweep was significantly reduced in the presence of Ca^{2+} and first latencies were clearly prolonged. Hence, P_s was corrected for the amount of sweeps with first latencies longer than the actual sweep length as determined from corresponding first latency distributions. The distribution determined under high (12–28 μM) Ca^{2+} has a proportion of 19.2%, which is above the sweep length of 475 ms (see Fig. 8 *A*). This means that in 19.2% of the sweeps the channel will not open within the sweep due to the Ca^{2+} effect on first latency, although

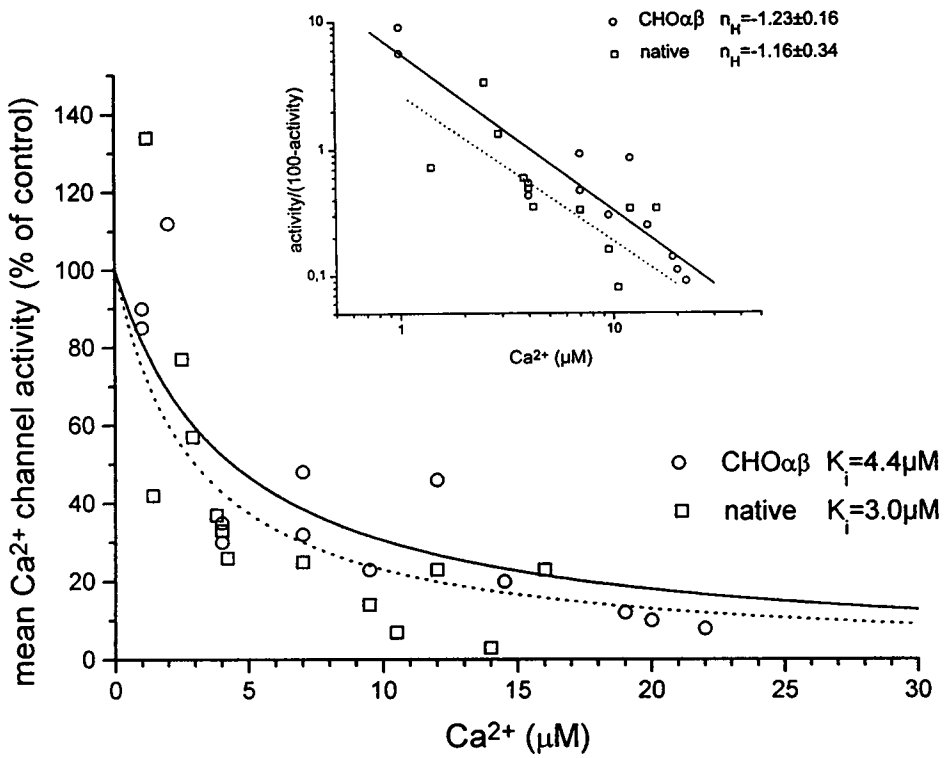

FIGURE 4 Ca^{2+} induces inactivation of native as well as expressed Ca^{2+} channels in a 1:1 interaction with a K_D of ~4 μM. The dependence of mean Ca^{2+} channel activity (percent of control) on the intracellular Ca^{2+} concentration is shown for native and expressed Ca^{2+} channels. The lines were obtained by fitting the data to $(N \cdot p)_{Ca}/(N \cdot p)_{control} = 1/(1 + Ca^{2+}/K_i)$, where $(N \cdot p)_{Ca}/(N \cdot p)_{control}$ refers to the probability of Ca^{2+} channels to remain open in the presence of intracellular Ca^{2+}. Thus, $(N \cdot p)_{Ca}/(N \cdot p)_{control} \times 100$ corresponds to percent of mean Ca^{2+} channel activity with 100% under control conditions (<20 nM Ca^{2+}). The inset contains the corresponding Hill plots with the Hill factors n_H (mean ± SD) calculated by linear regression of the logarithmically transformed data. The dashed line in both plots represents the fit to data from native cardiac Ca^{2+} channels. Results with expressed as well as native Ca^{2+} channels were obtained from 13 experiments in each case. A lack of response to Ca^{2+} was observed only in two experiments (5 and 8 μM Ca^{2+}) with expressed channels.

it would be available. Therefore, P_s under high (12–28 μM) Ca^{2+} was corrected for this first latency effect. In the presence of lower Ca^{2+} concentrations a correction of P_s was not necessary. Consequently, open probability at high (12–18 μM) Ca^{2+} concentrations was also adjusted, such as the overall activity $N \cdot P_s \cdot p_o$ remained constant. Introducing these corrections in Fig. 5 (*open symbols, dashed lines*) suggested that Ca^{2+} exerted its inhibitory effect predominantly by a reduction in channel p_o. A fit to the corrected p_o values (*smooth, dashed line*) yielded a K_i of 3.4 ± 1.4 μM Ca^{2+}, which is in good agreement with the K_i of 4.4 μM determined in Fig. 4 for the inhibition of $N \cdot p$.

Interestingly, we observed a large variation of Ca^{2+} channel p_o, but not of P_S, under control conditions. Therefore, we analyzed whether the effect of Ca^{2+} might depend on the channel's p_o, which could reveal a potential difference in the Ca^{2+} affinity of closed and open states obtained by the Ca^{2+} channel during depolarization. It is clearly evident in Fig. 7 that a severalfold difference in p_o did not significantly affect the amount of Ca^{2+} channel inactivation induced by three ranges of Ca^{2+} concentrations. Consistently, we did not observe a dependence of the Ca^{2+} effect on p_o when p_o was varied by different test potentials (n = 2, data not shown). Hence, binding of Ca^{2+} to the closed as well as open states of the channel during depolarization occurs apparently with similar affinity. Summarizing, Ca^{2+}-induced inactivation is predominantly caused by a reduction in p_o rather than P_s, involving a site with a K_i of ~4 μM. Next, we analyzed in detail how Ca^{2+} affected single channel kinetics leading to the diminished p_o.

Reduction in p_o is due to an increase in first latencies and a decrease in opening frequency as well as mean open times

For detailed analysis of the effect of Ca^{2+} on p_o we focused on Ca^{2+} concentrations in the higher range (12–28 μM) which reduce p_o to a similar extent of ~20% of control. In particular, we determined the effect of high intracellular Ca^{2+} on first latency, the frequency of the channel to open and on its mean open time. A summary of these results is shown in Fig. 8. First latency distribution under control conditions (<20 nM Ca^{2+}) exhibited two characteristic time constants, both of which were prolonged under high Ca^{2+} together with a moderate promotion in the proportion of the longer time constant (Fig. 8 *A*). Furthermore, the opening frequency of a channel within a depolarizing pulse was clearly reduced in the presence of high Ca^{2+}. This decrease was still prominent when the opening frequency was corrected for first latencies (Fig. 8 *B*, see Material and Methods). High Ca^{2+} also affected mean open times as shown in Fig. 8 *C*. Under control conditions (<20 nM Ca^{2+}) open time distribution was fitted by a biexponential function yielding two time constants, both of which were reduced in the presence of high Ca^{2+}. Fig. 8 *D* presents an overview regarding the extent by which an increase in first latency and a decrease in opening frequency as well as mean open time contributes to the reduction in p_o by high Ca^{2+}. The mean first latency calculated for a finite sweep length was increased from 68.6 ms under control conditions to 143.6 ms in the presence of high (12–28 μM) Ca^{2+}, yielding a relative reduction in p_o

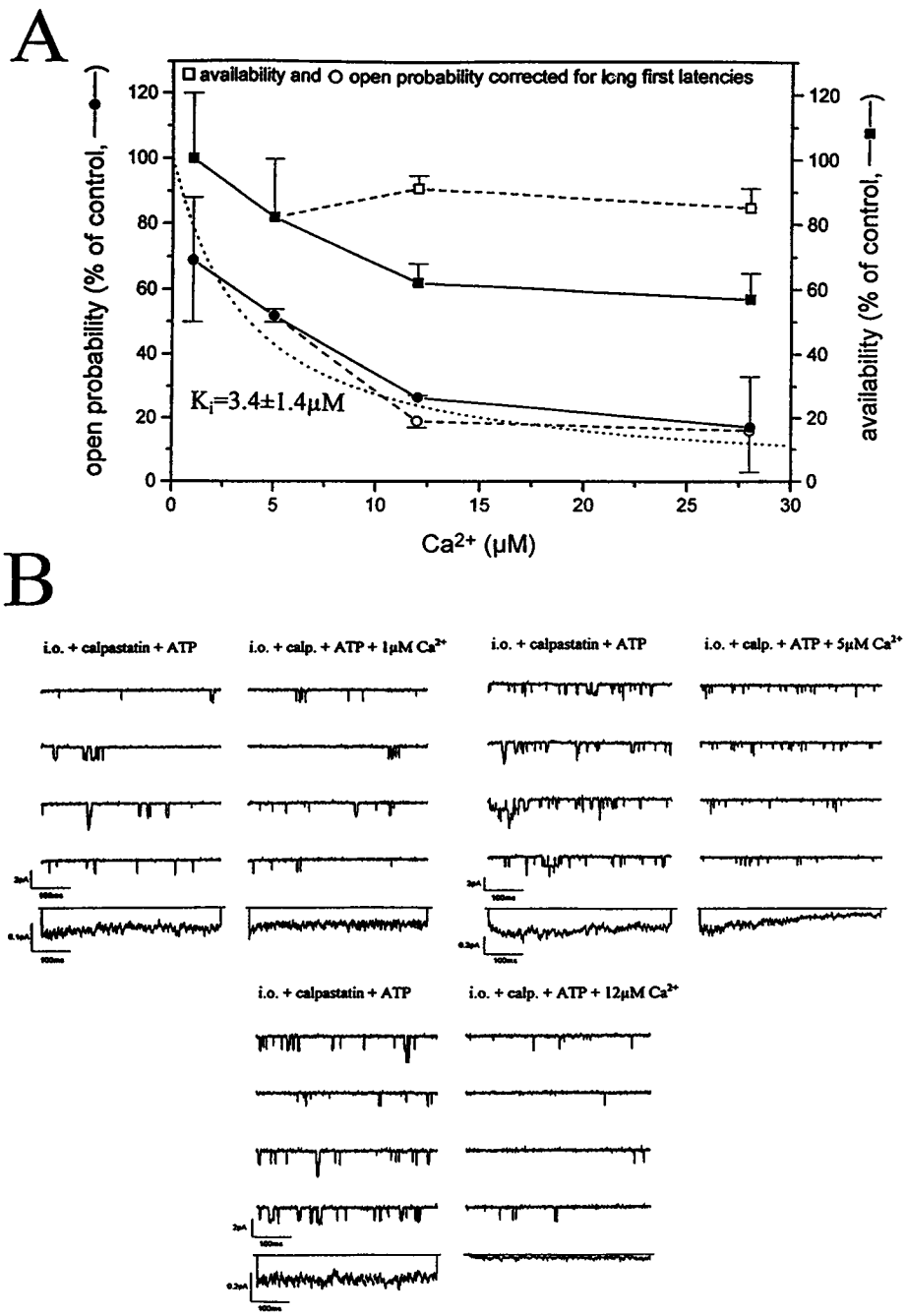

FIGURE 5 Ca^{2+} induces inactivation of Ca^{2+} channel activity of expressed $\alpha_{1C\text{-}a} + \beta_3$ subunits by a reduction of the channel's open probability rather than availability. Filled symbols depict the dependence of open probability as well as availability on the intracellular Ca^{2+} concentration. Open symbols connected by dashed lines represent values for open probability and availability, both corrected for long first latencies as described in the text. The smooth, dashed line corresponds to a fit (described in Fig. 4) of corrected values of open probability yielding a K_i of 3.4 ± 1.4 μM as derived from Hill plot analysis. Means ± SD are shown from two to four experiments at each Ca^{2+} concentration. (*B*) Single channel traces and their ensemble average currents (*bottom trace*) are shown for control conditions and in the presence of Ca^{2+} (1, 5, and 12 μM).

to 81%. This value was further corrected to account for the proportion of first latencies (19.2%, Fig. 8 *A*) longer than the sweep length resulting in an overall reduction in p_o to 64% of control caused by the relative increase in first latency. The decrease in the opening frequency corrected for mean first latencies as given above corresponded to a relative reduction in p_o to 45%. Comparison of mean open times under high Ca^{2+} (1.1 ms) and control conditions (1.7 ms) yielded a reduction in p_o to 64% of control. In total, the sum of kinetic alterations induced by Ca^{2+} was calculated by 0.64 × 0.45 × 0.64 leading to a calculated p_o of 18% of control, which is in close agreement with the determined p_o at high Ca^{2+}, as shown in Fig. 5, corroborating our analysis method. Hence, a relative proportion of 36:55:36 for the effect of first latency, opening frequency, and mean open time is responsible for a 82% suppression in p_o. Of this 82% suppression in p_o, 23% is due to an increase in first latency, 36% results from a decrease in opening frequency, and 23% occurs by a reduction in mean open time.

DISCUSSION

The main results presented here are that Ca^{2+} channel activity of expressed $\alpha_{1C\text{-}a} + \beta_3$ subunits, as well as of

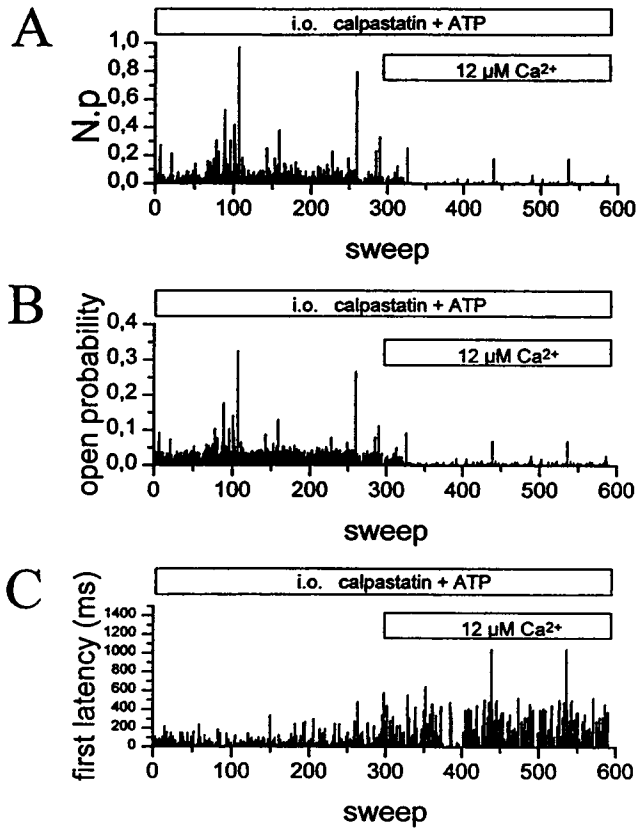

FIGURE 6 An increase in first latency contributes to the decrease in open probability by intracellular Ca^{2+}. (*A*) Diary plot of mean Ca^{2+} channel activity ($N \cdot p$) and corresponding plots of (*B*) open probability and (*C*) first latency, in the i.o. patch and following application of 12 μM Ca^{2+} as indicated. (*B*) and (*C*) were determined after calculation of the posterior probability of the number of channels in each sweep. The open probability in each sweep was calculated by dividing the respective channel activity through the estimated number of channels in this sweep. First latency in a sweep was corrected to that anticipated for a single channel by multiplying the time to first channel opening by the estimated number of channels in this sweep.

native Ca^{2+} channels, is suppressed by Ca^{2+} in a 1:1 molecular interaction with a binding site that exhibits a K_i of ~4 μM. This inactivation occurs mainly due to a reduction of the channel's p_o rather than P_s achieved by an increase in first latency and a decrease in opening frequency and mean open time.

Functional aspects of Ca^{2+}-induced inactivation

Our experiments demonstrated that intracellular Ca^{2+} in the μM range is able to inactivate Ca^{2+} channel activity without permeating the channel. Consistently, Ca^{2+}-dependent inactivation of unitary Ba^{2+} currents has been found with reconstituted cardiac Ca^{2+} channels at Ca^{2+} concentrations of 10–15 μM (Haack and Rosenberg, 1994). The observation that intracellular Ba^{2+} failed to mimic the effect of Ca^{2+} to induce inactivation unequivocally proved the specificity of the inactivation process for Ca^{2+}. Thus, the observed inhibitory effect is mediated by a binding site that exhibits selectivity for Ca^{2+} over Ba^{2+}. Binding of Ca^{2+} to this site apparently occurs with a similar affinity to the open as well as closed states of the channel during depolarization (0 mV) resulting in half-maximal inhibition at ~4 μM Ca^{2+}. The significant (50%) inactivation observed in ensemble average currents in this concentration range might be explained if the closed state reached during hyperpolarization (−80 mV) exhibits a lower Ca^{2+} affinity than the closed as well as open states obtained during depolarization. Inactivation manifested predominantly in a decrease in p_o rather than P_s substantiating separate mechanisms for Ca^{2+}- and voltage-dependent inactivation (Cavalie et al., 1986; Hadley and Lederer, 1991). In accordance, a Ca^{2+}-induced shift of channel gating from high to low open probability without an effect on channel availability has been recently proposed (Imredy and Yue, 1994) to yield a specific mode Ca^{2+} in native cardiac Ca^{2+} channels. Ca^{2+} in contrast to Ba^{2+} has been found (Imredy and Yue, 1994) to induce a more than 100-fold reduction of entry rate to the open state manifested by an additional, slow component in the first latency distribution and by a lower pedestal of the conditional open probability (P_{oo}). Correspondingly, a Ca^{2+}-induced increase in first latency and a decrease in the opening frequency was found in the present study. Furthermore, the reduction in mean open times as determined here might explain the more rapid decay as well as lower pedestal of P_{oo}. The presence of BAYK 8644 in our experiments might somewhat limit the relevance of the results with respect to natural gating. Nonetheless, our experimental approach subjecting Ba^{2+} currents of expressed $\alpha_{1C\text{-}a}$ + β_3 subunits to intracellular Ca^{2+} yielded qualitatively similar results regarding ion channel kinetics as the approach by Imredy and Yue (1994), who employed two pulse protocols

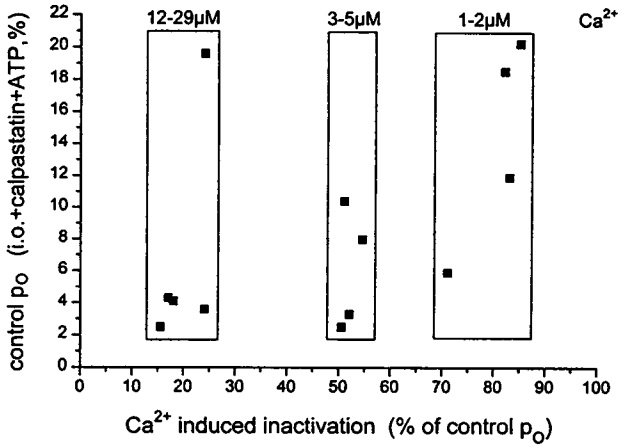

FIGURE 7 The extent of Ca^{2+}-induced inactivation does not depend on the preceding channel's p_o. The effect of three ranges of Ca^{2+} concentrations (1–2, 3–5, and 12–29 μM) is depicted in dependence of control p_o determined in the absence of Ca^{2+}. Ca^{2+}-induced inactivation is measured by the effect on p_o and is expressed in percent of control p_o. In the high concentration range (12–29 μM Ca^{2+}) p_o was corrected for the first latency effect on P_S as in Fig. 5.

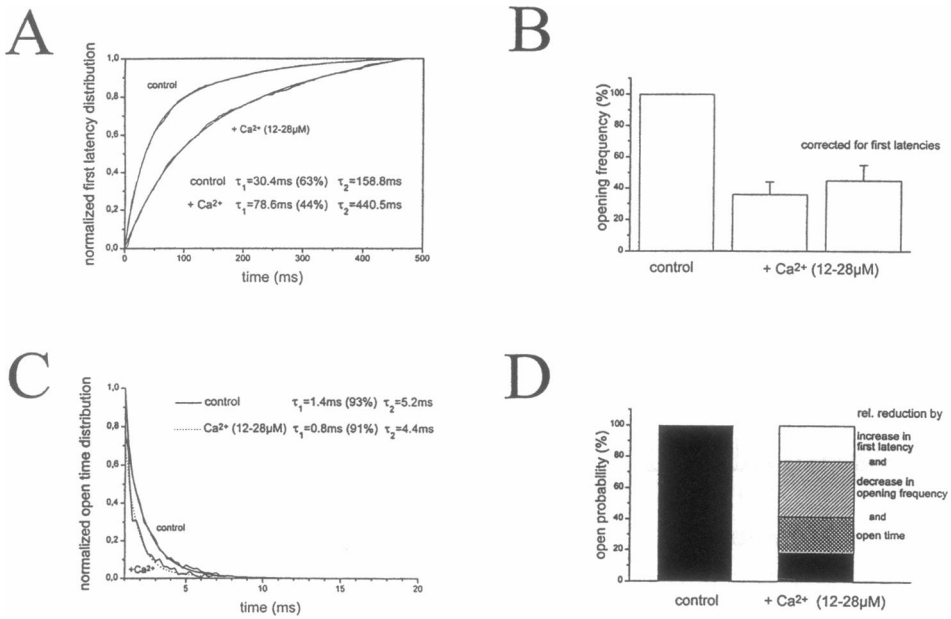

FIGURE 8 An increase in first latency and a decrease in the channel's opening frequency as well as mean open time contribute to the attenuation of open probability of expressed Ca^{2+} channels by intracellular Ca^{2+}. (*A*) Empirical first latency distributions normalized under control conditions and in the presence of Ca^{2+}. First latencies were accumulated from four experiments each with ~200 sweeps under control as well as Ca^{2+} conditions. Smooth lines represent first latency distributions obtained by a maximum likelihood algorithm yielding time constants and their relative proportions as indicated. (*B*) Relative reduction of the channel's opening frequency by Ca^{2+} determined with respect to the whole sweep length (*middle column*) and corrected for mean first latency (*right column*). (*C*) Normalized open time histograms for control conditions and under Ca^{2+}, which was scaled up by a factor of 3.3. Smooth lines were constructed by a biexponential fit of the data employing a maximum likelihood algorithm. Time constants and their relative proportions are indicated. (*D*) Ca^{2+} reduces open probability to 18% of control. The relative proportion of the effect of Ca^{2+} on first latency, opening frequency, and mean open time is shown. Results depicted in *A–C* are pooled data from three to four experiments. Mean ± SE is shown in *B*.

to induce inactivation of native cardiac Ca^{2+} channels in the absence of BAYK 8644.

Summarizing, Ca^{2+}-induced decrease in p_o is accomplished by stabilization of the closed state(s) of the channel and a destabilization of the open state(s), i.e., the Ca^{2+} channel opens less frequently and closes more rapidly. The application of steady-state Ca^{2+} concentrations in the present study enabled a quantitative evaluation of the proportion by which kinetic alterations contribute to the inactivation induced by Ca^{2+}. Specifically, a 44% reduction in the channel's opening frequency concomitant to a prolongation of first latency as well as a reduction of mean open time, both at a similar extent of 28%, contribute to the reduction in p_o at high (12–28 μM) Ca^{2+} concentrations. In conclusion, 72% of the Ca^{2+}-induced inactivation is mediated by a stabilization of the closed state(s) and 28% occurs due to a destabilization of channel open state(s).

Structural aspects of Ca^{2+}-induced inactivation

A similar K_i for the Ca^{2+}-induced inactivation as determined for expressed $\alpha_{1C\text{-}a} + \beta_3$ subunits and native cardiac Ca^{2+} channels demonstrated that the α_2/δ subunit might not significantly contribute to this process. The measured value of ~4 μM suggests a direct Ca^{2+} binding to the Ca^{2+} channel close to the channel's inner mouth, where such high Ca^{2+} concentrations would be easily reached during Ca^{2+} entry (Llinas et al., 1992) and Ca^{2+} release from the sarcoplasmic reticulum (Sipido et al., 1995). Two potential Ca^{2+}-binding domains have been proposed (Tanabe et al., 1987; Hescheler and Trautwein, 1988; Babitch, 1990) on the cytoplasmic regions of the α_1 subunit of the Ca^{2+} channel: an ultranegative locus of consecutive aspartate and glutamate residues in the linker between domains II and III, and an EF hand motif on the carboxy terminal close to the IVS6. The latter has been recently demonstrated (DeLeon et al., 1995) as an essential domain for Ca^{2+}-sensitive inactivation. Although a fair amount of scatter in the data of Fig. 4 does not allow to completely exclude Ca^{2+} binding to a second site, Hill plot analysis suggested a 1:1 molecular interaction of Ca^{2+} with its binding site, which would be in accordance with only one EF hand motif localized in the $\alpha_{1C\text{-}a}$ subunit. Consistently, EF hand-containing proteins become active as the Ca^{2+} concentration increases to 1–10 μM (Ikura, 1996), although most of the proteins usually contain at least two or four EF hand motifs.

Our characterization of the Ca^{2+}-induced inactivation process of expressed wild-type Ca^{2+} channel subunits is a prerequisite for a comparison with chimeric channels (Wang et al., 1996) with a proposed attenuation or lack of Ca^{2+}-sensitive inactivation. Quantitative evaluation of this inactivation based on determination of K_i and alterations of single channel kinetics would clearly enhance our under-

standing as to how Ca^{2+} affinity and selectivity are tuned in the EF hand motif of the Ca^{2+} channel.

We thank Dr. F. Sigworth for helpful discussions and B. Kenda and A. Schurga for their excellent technical assistance.

This study was supported by Austrian Research Funds S06605, S06606, S06607, and NB6121.

REFERENCES

Armstrong, D. L. 1989. Calcium channel regulation by calcineurin, a Ca^{2+}-activated phosphatase in mammalian brain. *Trends Neurosci.* 12: 117–122.

Babitch, J. 1990. Channel hands. *Nature.* 346:345–346.

Baumgartner, W., K. Hohenthanner, G. F. Höfer, K. Groschner, and C. Romanin. 1997. Estimating the number of channels in patch-clamp recordings: application to kinetic analysis of multichannel data from voltage-operated channels. *Biophys. J.* 72:1143–1152.

Biel, M., R. Hullin, S. Freundner, D. Singer, N. Dascal, V. Flockerzi, and F. Hofmann. 1991. Tissue-specific expression of a high-voltage-activated dihydropyridine-sensitive L-type calcium channel. *Eur. J. Biochem.* 200:81–88.

Carbone, E., and D. Swandulla. 1989. Neuronal calcium channels: kinetics, blockade and modulation. *Prog. Biophys. Mol. Biol.* 54:31–58.

Cavalie, A., D. Pelzer, and W. Trautwein. 1986. Fast and slow gating behavior of single Ca^{2+} channels in cardiac cells. Relation to activation and inactivation of calcium-channel current. *Pflügers Arch.* 406: 241–258.

Chad, J. E., and R. Eckert. 1986. An enzymatic mechanism for calcium current inactivation in dialyzed helix neurones. *J. Physiol.* 378:31–51.

Colquhoun, D., and F. J. Sigworth. 1995. Fitting and statistical analysis of single channel records. *In* Single-Channel Recording. B. Sakmann and E. Neher, editors. Plenum Press, New York. 483–587.

DeFelice, L. J. 1993. Molecular and biophysical view of the Ca channel: a hypothesis regarding oligomeric structure, channel clustering, and macroscopic current. *J. Membr. Biol.* 44:215–267.

DeLeon, M., Y. Wang, L. Jones, E. Perez-Reyes, X. Wei, T. W. Soong, T. P. Snutch, and D. T. Yue. 1995. Essential Ca^{2+}-binding motif for Ca^{2+}-sensitive inactivation of L-type Ca^{2+} channels. *Science.* 270: 1502–1506.

Eckert, R., and J. E. Chad. 1984. Inactivation of Ca channels. *Prog. Biophys. Mol. Biol.* 44:215–267.

Fabiato, A. 1988. Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solution containing multiple metals and ligands. *Methods Enzymol.* 157: 387–417.

Haack, J. A., and R. L. Rosenberg. 1994. Calcium-dependent inactivation of L-type calcium channels in planar lipid bilayers. *Biophys. J.* 66: 1051–1060.

Hadley, R. W., and W. J. Lederer. 1991. Ca^{2+} and voltage inactivate Ca^{2+} channels in guinea-pig ventricular myocytes through independent mechanisms. *J. Physiol.* 444:257–268.

Hamil, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp technique for high resolution current recordings from cell and cell-free patches. *Pflügers Arch.* 391:85–100.

Hescheler, J., and W. Trautwein. 1988. Modification of L-type Ca^{2+} current by intracellularly applied trypsin in guinea-pig ventricular myocytes. *J. Physiol.* 404:259–274.

Hofmann, F., M. Biel, and V. Flockerzi. 1994. Molecular basis for Ca^{2+} channel diversity. *Annu. Rev. Neurosci.* 17:399–418.

Hullin, R., D. Singer-Lahat, M. Freichel, M. Biel, N. Dascal, F. Hofmann, and V. Flockerzi. 1992. Calcium channel β subunit heterogeneity: functional expression of cloned cDNA from heart, aorta and brain. *EMBO J.* 11:885–890.

Ikura, M. 1996. Calcium binding and conformational response in EF-hand proteins. *TIBS.* 21:14–17.

Imredy, J. P., and D. T. Yue. 1992. Submicroscopic Ca^{2+} diffusion mediates inhibitory coupling between individual Ca^{2+} channels. *Neuron.* 9:197–207.

Imredy, J. P., and D. T. Yue. 1994. Mechanism of Ca^{2+}-sensitive inactivation of L-type Ca^{2+} channels. *Neuron.* 12:1301–1318.

Isom, L. L., K. S. DeJongh, and W. A. Catterall. 1994. Auxiliary subunits of voltage-gated ion channels. *Neuron.* 12:1183–1194.

Lacinova, L., A. Ludwig, E. Bosse, V. Flockerzi, and F. Hofmann. 1995. The block of expressed L-type calcium channel is modulated by the β_3 subunit. *FEBS Lett.* 373:103–107.

Llinas, R., M. Sugimori, and R. B. Silver. 1992. Microdomains of high calcium concentration in a presynaptic terminal. *Science.* 256:677–679.

McDonald, T. F., S. Pelzer, W. Trautwein, and D. J. Pelzer. 1994. Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells. *Physiol. Rev.* 74:365–507.

Neely, A., R. Olcese, X. Wei, L. Birnbaumer, and E. Stefani. 1994. Ca^{2+}-dependent inactivation of a cloned cardiac Ca^{2+} channel α_1 subunit (α_{1C}) expressed in *Xenopus* oocytes. *Biophys. J.* 66:1895–1903.

Romanin, C., P. Grösswagen, and H. Schindler. 1991. Calpastatin and nucleotides stabilize cardiac calcium channel activity in excised patches. *Pflügers Arch.* 418:86–92.

Romanin, C., J.-O. Karlsson, and H. Schindler. 1992. Activity of cardiac L-type Ca^{2+} channels is sensitive to cytoplasmic calcium. *Pflügers Arch.* 421:516–518.

Schmid, R., K. Seydl, W. Baumgartner, K. Groschner, and C. Romanin. 1995. Trypsin increases availability and open probability of cardiac L-type Ca^{2+} channels without affecting inactivation induced by Ca^{2+}. *Biophys. J.* 69:1847–1857.

Seydl, K., J.-O. Karlsson, A. Dominik, H. Gruber, and C. Romanin. 1995. Action of calpastatin in prevention of cardiac L-type Ca^{2+} channel run-down cannot be mimicked by synthetic calpain inhibitors. *Pflügers Arch.* 429:503–510.

Sherman, A., J. Keizer, and J. Rinzel. 1990. Domain model for Ca^{2+}-inactivation of Ca^{2+} channels at low channel density. *Biophys. J.* 58: 985–995.

Sipido, K. R., G. Gallewaert, and E. Carmeliet. 1995. Inhibition and rapid recovery of Ca^{2+} current during Ca^{2+} release from sarcoplasmic-reticulum in guinea-pig ventricular myocytes. *Circ. Res.* 76:102–109.

Tanabe, T., H. Takeshima, A. Mikami, V. Flockerzi, H. Takahashi, K. Kangawa, M. Kojima, H. Matsuo, T. Hirose, and S. Numa. 1987. Primary structure of the receptor for calcium channel blockers from skeletal muscle. *Nature.* 328:313–318.

Varadi, G., Y. Mori, G. Mikala, and A. Schwartz. 1995. Molecular determinants of Ca^{2+} channel function and drug action. *Trends Pharmacol. Sci.* 16:43–49.

Wang, Y., M. DeLeon, and D. T. Yue. 1996. EF hand region containing both sufficient and regulatory elements for Ca^{2+} inactivation of L-type Ca channels. *Biophys. J.* 70:238*a*. (Abstr.).

Welling, A., E. Bosse, A. Cavalie, R. Bottlender, A. Ludwig, W. Nastainczyk, V. Flockerzi, and F. Hofmann. 1993. Stable coexpression of calcium channel α_1, β, and α_2/δ subunits in a somatic cell line. *J. Physiol.* 471:749–765.

Yue, D. T., P. H. Backx, and J. P. Imredy. 1990. Calcium-sensitive inactivation in the gating of single calcium channels. *Science.* 250: 1735–1738.

Zong, X., and F. Hofmann. 1996. Ca^{2+}-dependent inactivation of the class C L-type Ca^{2+} channel is a property of the α_1 subunit. *FEBS Lett.* 378:121–125.