Essential role of the beta subunit in modulation of C-class L-type Ca^{2+} channels by intracellular pH

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Abstract Elevation of intracellular pH (pH_i) enhances the activity of native L-type Ca^{2+} channels in cardiac and smooth muscle. We studied the modulation by pH_i of expressed L-type Ca^{2+} channels comprised of either the α_{1C} subunits alone or of α_{1C} plus β_{2a} subunits. Ca^{2+} channels were expressed in human embryonic kidney cells (HEK 293) and pH_i was increased from a basal level of 7.3 to 8.3 by exposure of cells to NH_4Cl (20 mM) or by elevation of extracellular pH to 8.5. Elevation of pH_i enhanced the activity of Ca^{2+} channels derived by coexpression of α_{1C} and β_{2a} subunits. This alkalosis-induced stimulation of channel activity was mainly due to an increase in channel availability. Channels derived by expression of α_{1C} alone were not affected by intracellular alkalosis. Our results demonstrate that the pH_i sensitivity of L-type Ca^{2+} channels is conferred by the β subunit of the channel complex.

Key words: L-type Ca^{2+} channel; β Subunit; Intracellular pH; Cellular regulation

1. Introduction

Intracellular pH (pH_i) is known to control Ca^{2+} entry into cardiac and vascular smooth muscle [1–4]. One mechanism by which intracellular H^+ governs Ca^{2+} entry is the modulation of high voltage activated, L-type Ca^{2+} channels which provide a prominent Ca^{2+} entry pathway in cardiac and smooth muscle. It has been repeatedly demonstrated that the activity of L-type Ca^{2+} channels in cardiac and smooth muscle is inversely correlated with the intracellular H^+ concentration [1–3]. Ca^{2+} channel activity is determined by two principle single channel properties, (i) the probability of channels to open at least once during a depolarisation (availability, P_S), and (ii) the probability of single available channels to stay in an open state during the depolarisation (open probability, P_O). Analysis of the effect of pH_i on gating of L-type Ca^{2+} channels revealed a pH dependence of channel availability. A reduction in the intracellular H^+ concentration was found to enhance Ca^{2+} channel availability [2,3]. The molecular basis of this pH_i modulation of class C L-type Ca^{2+} channel function is still elusive. Determination of the apparent affinity of the H^+ acceptor site suggested an involvement of histidine residues [2], a large number of which are present in the long cytoplasmic carboxy terminus of the pore forming α1 subunit and in the hydrophilic β subunit of the channel.

This study was aimed at investigating the role of Ca^{2+} channel α_{1C} and β subunits in the modulation of L-type Ca^{2+} channels by pH_i. We present evidence that pH_i sensitivity of L-type Ca^{2+} channels is not an intrinsic property of the α_{1C} subunit but is conferred by the β subunit. Thereby we demonstrate an important physiologic and pathophysiologic function of the Ca^{2+} channel β subunit in cardiac and smooth muscle.

2. Materials and methods

2.1. Cell culture and transfection

HEK 293 were stably transfected with the coding region of the α_{1C-b} subunit (HEKα_{1C-b}) as described [5]. To study the function of channels comprised of α_{1C-b} [6] and β_{2a} [7] subunits, HEKα_{1C-b} were transiently transfected as described in [8] with β_{2a} subunit together with green fluorescent protein (GFP) at a DNA ratio of 4:1. GFP fluorescence was used as a marker to identify cells which were successfully transfected. Alternatively, both α_{1Cb} and β_{2a} subunits were transiently transfected into HEK 293 using a 4 fold excess of the β encoding plasmid. Some experiments were performed with CHO cells stably transfected with the coding region of the α_{1C-a} [9] together with the β_3 [7] subunit as described in [10]. HEK 293 and CHO cells were cultured in DMEM and HAM's F12, respectively. Both media were supplemented with 10% FCS and contained 0.178 g/l geneticin when used for culture of stably transfected cells.

2.2. Measurement of intracellular pH

Intracellular pH was determined using the fluorescent pH indicator BCECF, as described [11]. In brief, cells were suspended in 5 ml of physiological solution containing [mM]: 137 NaCl, 5 KCl, 2.5 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, pH 7.4, and loaded with BCECF for 60 min at 37°C. After loading, the cells were once washed and resuspended in high K^+ low Cl^- bath solution (see below). Fluorescence measurements were carried out using a dual wavelength spectrophotofluorometer (Hitachi F2000). The cell suspension (2 ml) was stirred and maintained at room temperature. Excitation wavelengths were 506 nm and 455 nm, and emission was collected at 530 nm. Calibration of pH_i measurements were performed using nigericin (7 μM)-containing high K^+ solution at various pH_e [11].

2.3. Current measurements

Ba^{2+} currents through single Ca^{2+} channels were recorded in the membrane of intact cells (cell-attached mode). Cell potential was set to approximately zero by use of a high K^+ low Cl^- bath solution which contained [mM]: 110 K^+ aspartate, 20 KCl, 2 $MgCl_2$, 20 HEPES, 2 EGTA, pH was adjusted to 7.4 or 8.5 with *N*-methyl-D-glucamine, and pCa was adjusted to 7 [12]. The pipette solution contained [mM]: 10 $BaCl_2$, 100 NaCl, 30 TEA-Cl and 15 HEPES, pH adjusted to 7.4 with *N*-methyl-D-glucamine. Since channel activity in controls was usually too low to allow for a proper analysis of channel gating, experiments were performed in presence of the dihydropyri-

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Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethansulfonic acid; pH_e, extracellular pH; pH_i, intracellular pH; P_O, a channel's probability of being in the open state; P_S, a channel's probability to open upon a depolarisation

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dine-Ca^{2+} channel activator S(−)-BayK 8644 (0.5 μM) which was added to the pipette solution. Patch pipettes were fabricated from borosilicate glass (Clark Electromedical Instruments; Pangbourne UK), and had resistances of 5–10 MΩ. All experiments were performed at room temperature.

Voltage clamp and current amplification was performed with a List EPC/7 patch-clamp amplifier (List, Darmstadt, Germany). For data analysis, current records were filtered at 1 kHz (−3 dB) and digitised at a rate of 5 kHz. For display, current records were filtered at 500 Hz (−3 dB) and digitised at a rate of 2 kHz. Voltage protocols were controlled with pClamp software (Axon Instruments, Foster City, CA, USA). For idealisation of current records a custom made level detection software was employed [13]. Ca^{2+} channel activity was calculated as the mean number of open channels during depolarising pulses, and the gating properties of single channels were analysed in terms of channel availability (P_S), i.e. the probability that a channel will open upon depolarisation, as well as the open probability (P_O) of available channels as described previously [14,15]. Averaged data are given as mean ± S.E.M. from the indicated number of experiments. Statistical analysis was performed using Student's *t*-test for paired values and differences were considered statistically significant at $P < 0.05$.

2.4. Materials

Tissue culture media were from Gibco BRL (Vienna, Austria), and BCECF from Lambda Probes (Graz, Austria); S(−)-BayK 8644 was from Research Biochemicals Incorporated (Natic, MA, USA) and all other chemicals from Sigma Chemical Co. (Vienna, Austria). A green fluorescent protein construct (pCMV2/hGFP) was kindly provided by Dr. Ed C. Conley (Leicester, GB).

3. Results and discussion

3.1. L-type Ca^{2+} channels comprised of α_{1C-b} plus β_{2a} subunits are sensitive to intracellular pH

Modulation of expressed L-type Ca^{2+} channels by intracellular pH (pH_i) was investigated by recording channel activity in the cell-attached configuration. Two manoeuvres were used to elevate intracellular pH in HEK 293 cells: (i) elevation of extracellular pH (pH_e) from 7.4 to 8.5 and (ii) exposure of cells to extracellular NH_4Cl (20 mM). Both interventions resulted in a rapid increase of pH_i from a resting level of 7.3 to about 8.3 (Fig. 1). It has been previously demonstrated that such elevation of pH_i causes a marked stimulation of L-type Ca^{2+} channel activity in cardiac [2] and smooth muscle tissue [3]. Consistently, we observed (Fig. 2) that class C L-type Ca^{2+} channels derived by expression of α_{1c-b} and β_{2a} subunits were substantially stimulated during intracellular alkalisation. Fig. 2A illustrates typical time courses of channel activity in cell-attached patches during administration of NH_4Cl (20 mM, at pH_e 7.4; upper panel) and a high pH (pH_e 8.5) bath solution (lower panel). Stimulatory effects reversed during subsequent perfusion with standard bath solution (pH_e 7.4). It is of note that, in the cell-attached recording configuration, the bath solutions do not have access to the extracellular side of the Ca^{2+} channels, excluding changes of pH at the extracellular face of the channel. Fig. 2B illustrates Ca^{2+} channel activity recorded at control conditions corresponding to a pH_i of 7.3 and at an elevated pH_i of 8.3. Average Ba^{2+} currents increased clearly when pH_i was elevated by either NH_4Cl ($N = 6$) or a pH_e of 8.5 ($N = 5$). These results demonstrate that Ca^{2+} channels directed by expression of the α_{1c-b} and the β_{2a} subunits exhibit sensitivity to pH_i similar to that of native Ca^{2+} channels in cardiac and smooth muscle cells [2,3]. A typical feature of pH_i regulation of native L-type Ca^{2+} channels is the prominent effect of pH_i on channel availability [2,3]. We have therefore analysed the modulation of Ca^{2+} channels derived by expression of α_{1C-b} and β_{2a} subunits by intracellular alkalosis in terms of changes in the channels' open probability and availability.

3.2. Elevation of intracellular pH increases availability of expressed α_{1C-b} plus β_{2a} channels

Since in most experiments (14 out of 15 patches) multiple channels were detected, we employed a recently developed method for the determination of the total number, P_O and P_S of channels from multi-channel records [14,15]. Fig. 3A illustrates Ca^{2+} channel activity recorded at basal and elevated pH_i (8.3) from a patch which was found to contain two channels exhibiting a mean P_O of 4% and a mean Ps of 20%. This situation results in a basal channel activity which is characterised by the occurrence of mainly one open level and an appreciable frequency of blank sweeps reflecting the coincidence of both channels being unavailable. As shown in Fig. 3A, the most striking effect of intracellular alkalosis was a reduction of the occurrence of blank sweeps indicating that elevation of pH_i increases P_S. Fig. 3B shows the mean values of P_O and P_S obtained in experiments using NH_4Cl to induce elevation of pHi, demonstrating that intracellular alkalosis indeed enhanced preferentially P_S. These results are in keeping with previous reports on pH_i modulation of native cardiac [2] and smooth muscle L-type Ca^{2+} channels [3] and suggest that expressed channels which are comprised of α_{1C-b} and β_{2a} subunits exhibit a similar pH_i-dependent gating behaviour as native L-type Ca^{2+} channels of cardiac and smooth muscle.

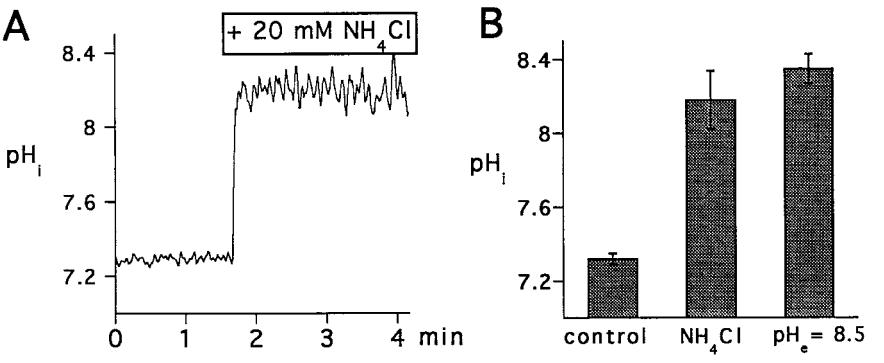

Fig. 1. Elevation of pH_i in HEK 293 cells by administration of NH_4Cl (20 mM) or elevation of pH_e to 8.5. (A) Representative time course of pH_i measured by BCECF fluorescence before and after acute intracellular alkalosis induced by 20 mM NH_4Cl. (B) Mean values ± S.E.M. ($N = 4$) of pH_i measured in standard bath solution (control), in the presence of 20 mM NH_4Cl, and in extracellular solution of pH 8.5 as indicated.

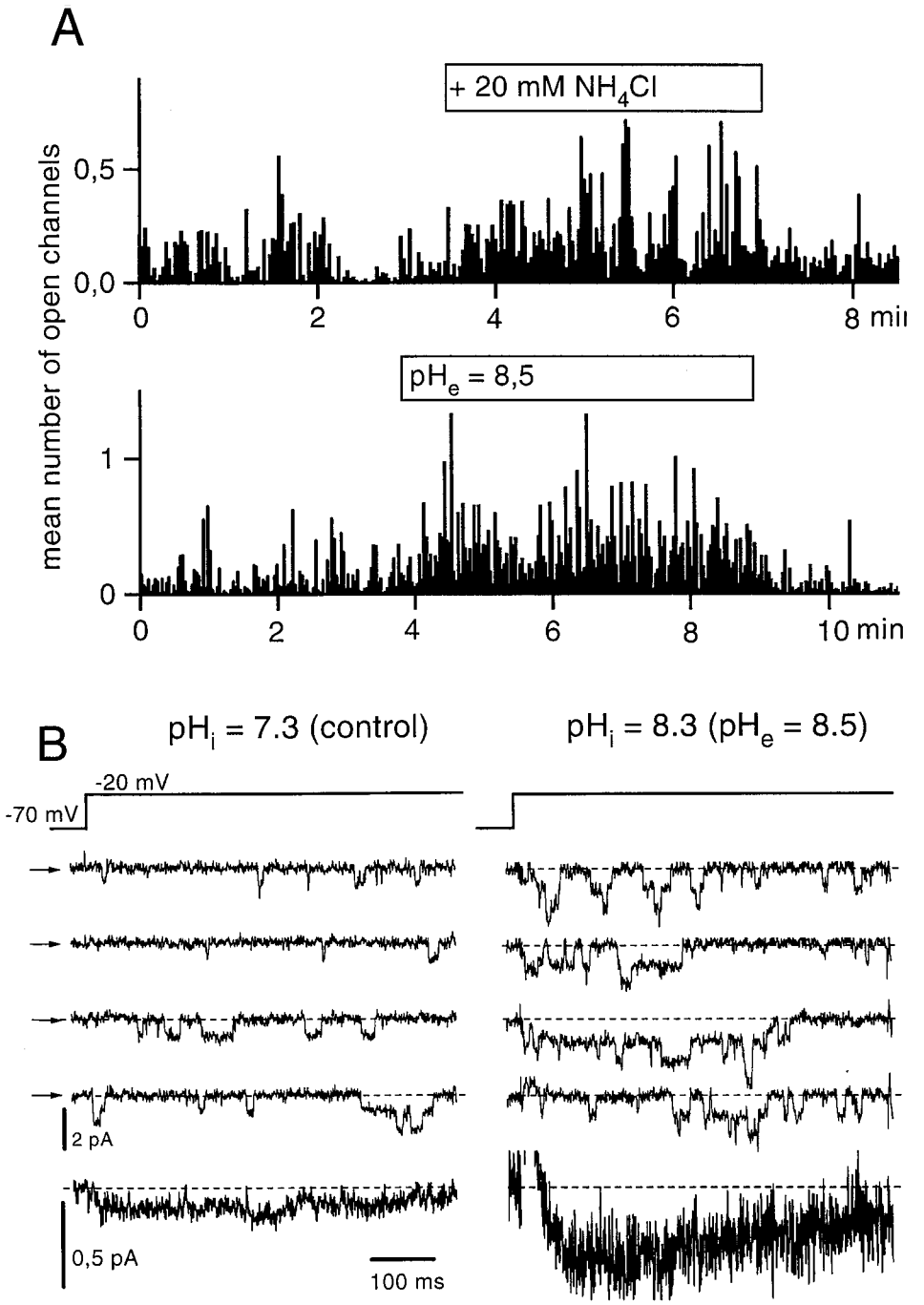

Fig. 2. L-type Ca^{2+} channels derived by expression of α_{1C-b}*β_{2a} subunits are stimulated during intracellular alkalosis induced by addition of NH_4Cl (20 mM) or elevation of pH_e to 8.5. **A:** Time courses of channel activity given as the mean number of open channels during individual depolarising voltage steps (holding potential −70 mV, test potential −20 mV, duration 500 ms, rate 0.66 Hz). Administration of NH_4Cl and elevation of extracellular pH to 8.5 are indicated. (B) Four consecutive current responses and corresponding ensemble average currents obtained from 170 and 150 depolarisations recorded at pH_i 7.3 (control) and at pH_i 8.3 (pH_e of 8.5). Records are filtered at 500 Hz and digitised at 2 kHz. Zero current levels are indicated by arrows or dashed lines.

3.3. *The β subunit is essential for pH_i sensitivity of class C L-type Ca^{2+} channels.*

Ca^{2+} channels directed by expression of α_{1C-b} subunits alone were not affected by elevation of pH_i to 8.3. Fig. 4A shows a typical recording of channel activity in a cell transfected with α_{1C-b} only. Exposure of the cell to 20 mM NH_4Cl failed to increase α_{1C-b} channel activity. Fig. 4B summarises the experiments with NH_4Cl in cells transfected with α_{1C-b} and in cells cotransfected α_{1C-b} plus β_{2a}. In all of 7 experiments using NH_4Cl (20 mM) and in 5 experiments using elevation of pH_e (8.5) for induction of intracellular alkalosis, α_{1C-b} channels lacked the typical pH_i sensitivity, suggesting that the β subunit is essential for pH_i-dependent upregulation of expressed L-type Ca^{2+} channels. The β subunit of L-type Ca^{2+} channels is known to modulate fundamental properties of the channel such as voltage-dependent activation and in-

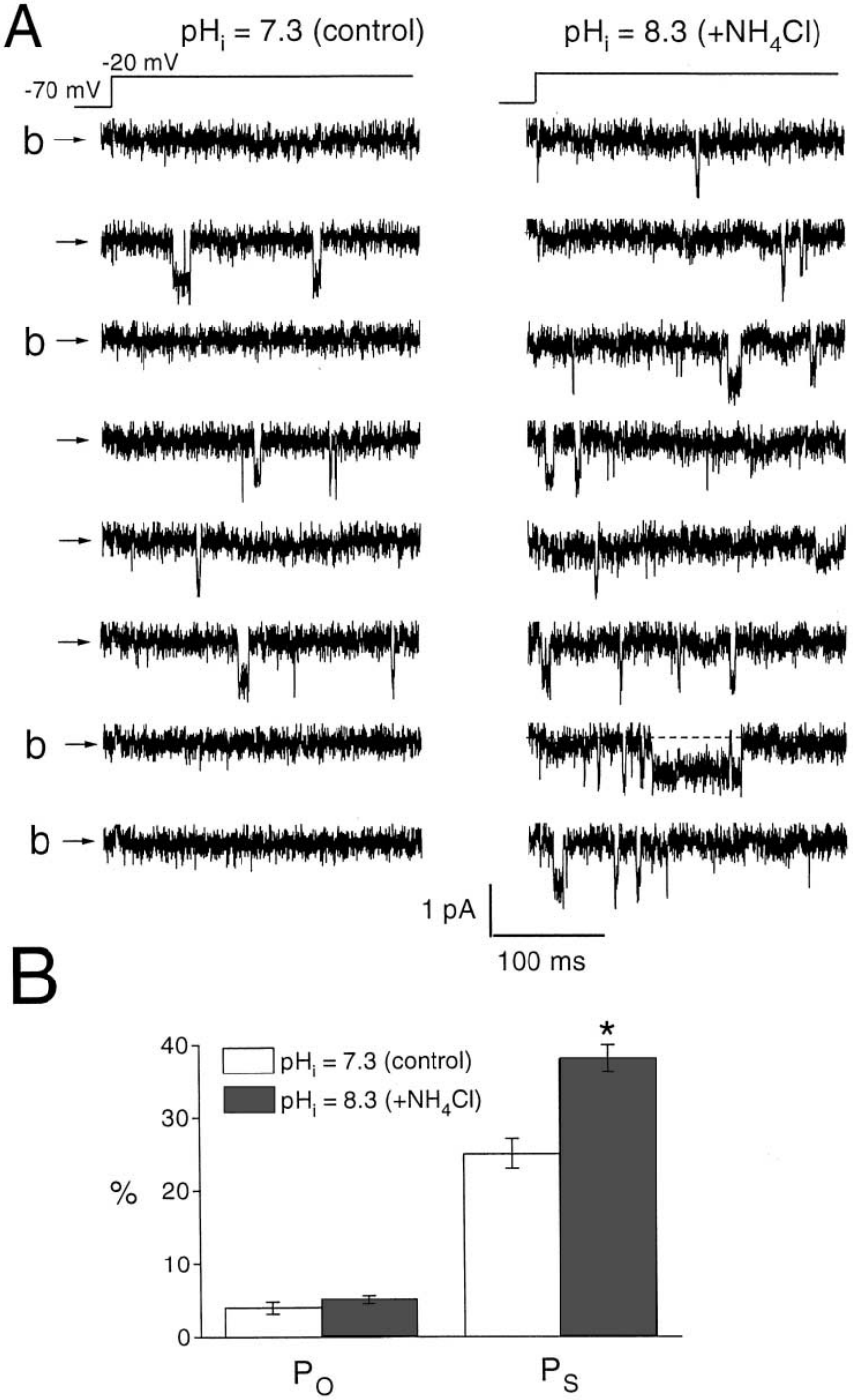

Fig. 3. Intracellular alkalosis enhances the availability of single α_{1C-b}*β_{2a} channels. (A) Consecutive current traces (holding potential −70 mV, test potential −20 mV, duration 500 ms, rate 0.66 Hz) at pH$_i$ 7.3 (control) and at pH$_i$ 8.3 (induced by NH_4Cl). Closed state is indicated by arrows or dashed lines respectively. Blank sweeps (b) are marked. Records are filtered at 500 Hz and digitised at 2 kHz. (B) Mean values ± S.E.M. ($N=5$) of P_O (open probability of a single available channel during a depolarisation) and P_S (availability) for control (pH$_i$ 7.3) and elevated pH$_i$ (pH$_i$ 8.3, induced by NH_4Cl). Asterisk denotes statistically significant difference versus control ($P<0.05$).

activation [7,16,17]. These characteristic features of L-type channels are in principle properties of the pore forming α_1 subunit but are substantially modified by interaction of this central channel subunit with the auxiliary β subunit. Recently, evidence has been accumulated that a prominent mechanism of cellular regulation, i.e. Ca^{2+}-dependent inactivation is an intrinsic property of the α_1 subunit [18,19]. The present results identify for the first time a regulatory property of the L-type Ca^{2+} channel, i.e. pH$_i$ sensitivity, which is exclusively dependent on expression of the β subunit.

To date four different isoforms of Ca^{2+} channel β subunits have been cloned [4] and significant differences in their modulatory interaction with α_1 subunits have been observed [17,20]. We have therefore tested whether another β subunit which is likely to be expressed in cardiac and vascular tissue, i.e. β_3 [7] is as well able to confer pH$_i$ sensitivity to α_{1C}

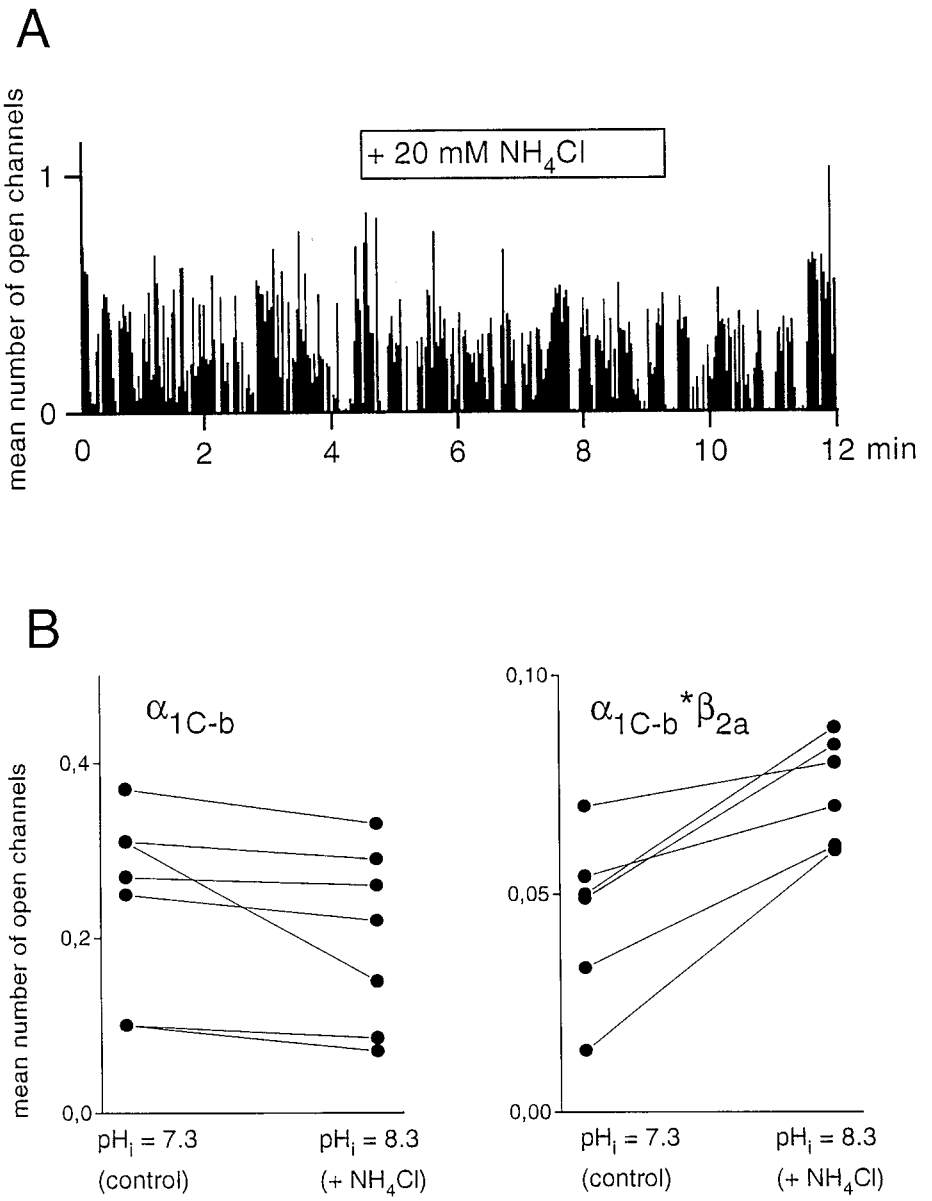

Fig. 4. Expression of the β subunit is required for sensitivity of L-type Ca^{2+} channels to pH_i. (A) Time course of channel activity given as the mean number of open channels during individual depolarising voltage steps (holding potential −70 mV, test potential −20 mV, duration 500 ms, rate 0.66 Hz). The cell was transfected with α_{1C-b}, administration of 20 mM NH_4Cl is indicated. (B) Changes in channel activity induced by intracellular alkalosis elicited by NH_4Cl in individual cells transfected with either α_{1C-b} or with α_{1C-b} plus β_{2a}.

channels. In 3 experiments performed with CHO cells stably expressing α_{1C-a} plus β_3 subunits, the channels were found to respond to elevation of pH_i with a clear increase in activity (data not shown). It appears reasonable to conclude that the interaction of α_{1C} subunits with either β_{2a} or β_3 subunits is a prerequisite to enable pH_i modulation of channel availability.

Contractility and pH_i of vascular and cardiac myocytes are regulated by a variety of hormones and neurotransmitters and both parameters are known to be substantially affected in pathological states such myocardial ischemia [21,22]. Modulation of class C L-type Ca^{2+} channel gating by pH_i appears to be one important mechanism for the control of vascular and cardiac contractility in terms of physiologic regulation but also pathophysiologic dysfunction. Thus, our present results strongly suggest that Ca^{2+} channel β subunits, by conferring the property of pH_i sensitivity to α subunits, play a critical role in cardiovascular physiology and pathophysiology.

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