The β subunit controls the gating and dihydropyridine sensitivity of the skeletal muscle Ca^{2+} channel

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ABSTRACT The skeletal muscle (SKM) L-type Ca^{2+} channel is composed of a central subunit designated α_1, which contains the pore and the dihydropyridine (DHP) binding domains and three associated subunits, $\alpha 2/\delta$, β, and γ, which influence the activity of the SKMα1. Coexpression of SKMα1 and SKMβ in stably transfected mouse L cells results in a dramatic increase in DHP binding accompanied by fast gated Ba^{2+} currents. We report here that this "SKM$\alpha_1\beta$-related phenotype" can be converted upon intracellular trypsin treatment into a slowly inactivating, DHP sensitive "SKMα1 phenotype." These observations indicate that current amplitude, fast inactivation, and DHP sensitivity are modulated by an interaction of SKMα1 and SKMβ on the internal side of the membrane.

INTRODUCTION

Complementary DNAs coding for the SKM Ca^{2+} channel subunit proteins have been cloned (1–4). The SKMα1 subunit generates a high voltage activated, very slowly inactivating, and DHP-sensitive Ca^{2+} channel when expressed in mouse L cells (5). The associated $\alpha 2/\delta$, β, and γ subunits have putative regulatory characteristics (6–12) that can be described as: (1) facilitation of anchoring of α1, (2) protein stabilization and (3) direct subunit modulation. Thus far, no clear evidence for any of these mechanisms has emerged. We now report that an interaction between SKMα1 and SKMβ is specifically disrupted by intracellular proteolytic treatment, demonstrating an essential role for the β subunit in SKM Ca channel gating and DHP modulation.

MATERIALS AND METHODS

In mouse Ltk^- cells, a cell line was established, expressing the SKM α1 subunit (LCa.11) as previously described (5). The parental Ltk^- cell line displayed no detectable Ca^{2+} channel activity, as well as transcripts for any of the SKM Ca^{2+} channel subunit (5, 6). LCa.11 cells were transfected with various combinations of expression plasmids for SKMα2, SKMβ, and SKMγ subunit complementary DNAs as previously described (6). Stable transformation by cDNA transfection, selection of surviving cell colonies, and Northern blot analyses were performed as previously described (6), in order to characterize LCa.11-derived cell lines coexpressing Ca^{2+} channel subunits. Two cell lines, coexpressing only SKMα1 and SKMβ (L$\alpha 1\beta$/6, L$\alpha 1\beta$/7), and a cell line coexpressing SKMα1, SKMβ, SKMα2, and SKMγ (L$\alpha 1\alpha 2\beta\gamma$/3) were used in these experiments. Dot blot and Northern blot analyses were used to quantitate the approximate level of SKM subunit mRNA in the various transfectants, as described in (13). The relative mRNA expression levels were estimated in cell lines coexpressing SKMα1 and SKMβ. The level of SKMα1 mRNA remained identical in the LCa.11 and all derived cell lines. Although we do not know the exact correlation between mRNA ratios and protein ratios in these cell lines, we might estimate that relative protein expression levels were kept in an array similar to mRNA ratios.

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The methods for electrophysiological recordings were previously described (6). All the Ca^{2+} channel current measurements were done using 40 mM Ba^{2+} in the external medium (mM): $Ba(OH)_2$, 40; glutamate, 40; *N*-methyl D-glucamine, 80; HEPES, 10; $MgCl_2$, 2 (pH adjusted to 7.4 with CH_3SO_3H). The analyzed cells had membrane capacitances between 80 and 120 pF. Three different trypsin preparations were tested with identical results (type I, type XI, both from Sigma Chemical Co., St. Louis, MO; trypsin 2.5%, Gibco Laboratories, Grand Island, NY). Most of the experiments were done using a trypsin concentration of 1 mg/ml (see, for example, reference 17). At higher concentration (2 mg/ml) the effects occurred even faster. Other enzymes used were purchased from Sigma Chemical Co.: carboxypeptidase A (type I; 1 mg/ml), leucine aminopeptidase (type III; 250 IU/ml). These enzymes were added to a standard pipette solution (mM): *N*-methyl D-glucamine, 110; $MgCl_2$, 2; EGTA, 20; HEPES, 15 (pH adjusted to 7.3 with CH_3SO_3H). The protease-containing solutions were freshly prepared (renewed every 2 h) and filtered before filling the pipette. No effect on Ba^{2+} current modulation was observed when trypsin was inactivated by heat or used in the presence of specific inhibitors (not shown).

RESULTS AND DISCUSSION

It has been shown for Na^+ channels (14), *Shaker* K^+ channels (15, 16), as well as Ca^{2+} channels (17–19), that major structural determinants involved in ion channel inactivation are accessible to internally perfused proteases. In L$\alpha 1\beta$/6 cells coexpressing the SKMα1 and SKMβ subunits, internal perfusion with trypsin amplified Ba^{2+} current by three- to fourfold (Fig. 1 *a*). This time-dependent increase in Ba^{2+} current amplitude was associated with a slowing of the current kinetics (n = 11). After 5 to 7 min of trypsin treatment, the inactivation of the Ba^{2+} currents became similar to that obtained in cells expressing SKMα1 alone (Fig. 1 *b;* reference 6). A similar result was obtained upon internal perfusion with carboxypeptidase A, since the treatment with this protease also increased current amplitude and slowed current inactivation (n = 3; Fig. 1 *c*). In contrast, leucine aminopeptidase (n = 4) produced no change in the Ba^{2+} current properties (data not shown). Extracellular application of trypsin (1 mg/ml, for 5 min) was also ineffective (n = 3).

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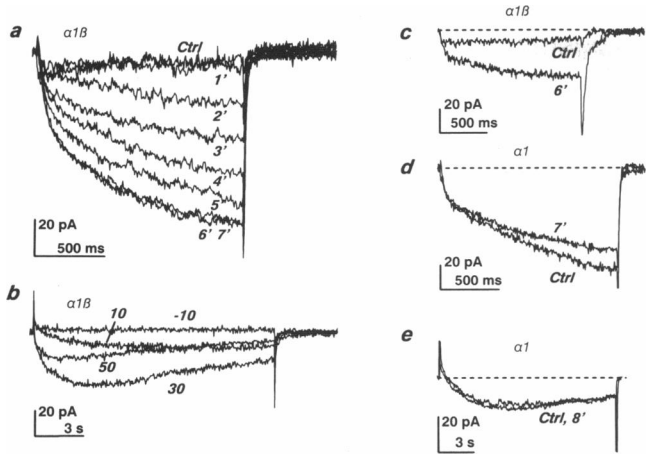

FIGURE 1 Differential effects of internal perfusion of proteases in LCa.11 cells and Lα1β/6 cells. Ba^{2+} currents were recorded on Lα1β/6 cells (*a, b, c*) using a +20 mV test pulse (TP; *a, c*) from −60 mV holding potential (*HP*). A +30 mV TP was used to record α1-related Ba^{2+} currents on LCa.11 cells (*d, e*). (*a*) Ba^{2+} currents were recorded every min for 7 min (as indicated on the traces) upon intracellular perfusion of trypsin (1 mg/ml) in Lα1β/6. (*b*) Family of Ba^{2+} currents recorded on Lα1β/6 cell perfused for 8 min with 1 mg/ml trypsin. The TPs are indicated on the traces. The threshold of activation was observed for a TP at 10 mV. The peak of the current-voltage relationship occurred at 30 mV (time to peak: 3.4 s). (*c*) Effect of internal perfusion with carboxypeptidase A (1 mg/ml) in a Lα1β/6 cell (Ctrl and after 6 min). 1 μM Bay K 8644 was present in the bath. (*d*) Time dependent modulation of the Ba^{2+} current upon trypsin perfusion in a LCa.11 cell (Ctrl and after 7 min). (*e*) Effect of carboxypeptidase A perfusion in a LCa.11 cell (Ctrl and after 8 min).

The effects of intracellular trypsin treatment on current amplitude and inactivation observed in Lα1β/6 cells resemble those reported for cardiac and smooth muscle L-type Ca^{2+} channels (17–19), an effect which has been attributed to proteolytic action on the α1 subunit (17). Contrary to this concept, intracellular perfusion of trypsin (1 mg/ml) in cells expressing only the SKMα1 subunit (LCa.11), produced no change in amplitude or kinetics of the Ba^{2+} current (n = 17; data not shown). Moreover, in some of these cells, a rundown (Fig. 1 *d*) of the SKMα1 Ba^{2+} current amplitude was observed (35 ± 9% decrease in Ba^{2+} current amplitude n = 5, after 5 min; less than 5 to 10% in control cells, n = 20). The SKMα1 Ba^{2+} current was also insensitive to other proteases such as carboxypeptidase A (1 mg/ml; n = 5; Fig. 1 *e*) that increase native Ca^{2+} channel currents (17). Similarly, in Lα1α2/3 cells (see reference 6), which coexpress SKMα1 and SKMα2, intracellular perfusion of trypsin (1 mg/ml) did not affect the Ba^{2+} current properties. These observations indicate that $SKM\alpha_1$ functions are unaffected by proteolytic cleavage. Taken together, the data clearly show that structural determinants of SKM Ca^{2+} channel gating must involve both the α_1 and the β subunits.

Coexpression of SKMα1 and SKMβ resulted in Ba^{2+} currents with greatly reduced sensitivity to the DHP agonist Bay K 8644 (Fig. 2 *a;* reference 6). After trypsin treatment of Lα1β/6 cells, the Ba^{2+} current displayed sensitivity to Bay K 8644 (Fig. 2 *b*) as well as to the DHP antagonist isradipine (PN200-110) (data not shown). The tail current amplitude was also enhanced in the presence of Bay K 8644 (Fig. 2 *b*), similar to that reported for cells expressing α1 alone (5, 6). The restoration of Bay K 8644 enhancement of the tail current required 4 to 6 min of trypsin treatment (Fig. 2 *c*), comparable to the time-dependent increase in current amplitude (Fig. 1 *a*). Ba^{2+} currents were greatly enhanced by the presence of 1 μM Bay K 8644 (2.5-fold, n = 7), and displayed the characteristic leftward shift of the I/V relationship of 18 ± 3 mV (n = 7) (Fig. 2 *d*). These data indicate that in trypsin-treated Lα1β/6 cells, "DHP pharmacology" became the same as that observed in LCa.11 cells (i.e., expressing the α1 subunit alone).

In a parallel set of experiments, we quantitated the increase in Ba^{2+} current amplitude by Bay K 8644 in cell lines coexpressing SKMα1 and SKMβ in various mRNA ratios. Cell lines Lα1β/7 and Lα1α2βγ/3 expressed Ba^{2+} currents with fast kinetics (Fig. 3, *a* and *b*), as previously described for the Lα1β/6 and Lα1βγ/1 cell lines (6). The stimulatory effect of Bay K 8644 on the expressed Ba^{2+} current amplitude was reduced in all cell lines expressing the β subunit. It is of interest to note that an increase in the expression of SKMβ is paralleled with a decrease in Bay K 8644 enhancement of Ba^{2+} current amplitude (Fig. 3 *c*). Thus, the β subunit appears to regulate the DHP sensitivity of the SKM Ca^{2+} channel.

Our data provide evidence that the SKMβ modulation

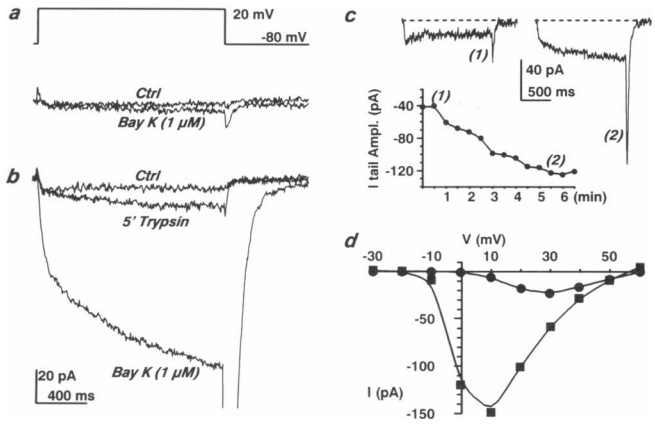

FIGURE 2 Recovery of DHP sensitivity after proteolytic treatment of Lα1β/6 cells. (*a*) Sensitivity to 1 μM Bay K 8644 (TP + 20 mV, HP − 60 mV) of an α1β-related Ba^{2+} current recorded in Lα1β/6 cell in control condition. (*b*) In the same batch of Lα1β/6 cells, increase in amplitude and slowing of the Ba^{2+} current after perfusion of trypsin (1 mg/ml). Note that subsequent application of 1 μM Bay K 8644 in the bath increased the Ba^{2+} current amplitude. (*c*) Time dependence of Bay K 8644-mediated increase of tail current amplitude in a Lα1β/6 cell perfused with trypsin (1 mg/ml). 1 μM Bay K 8644 was present in the bath. The tail currents after 30 s (*1*) and 330 s (*2*) are presented. (*d*) Comparison of current-voltage relations (*filled circles:* Ctrl; *filled squares:* 1 μM Bay K 8644).

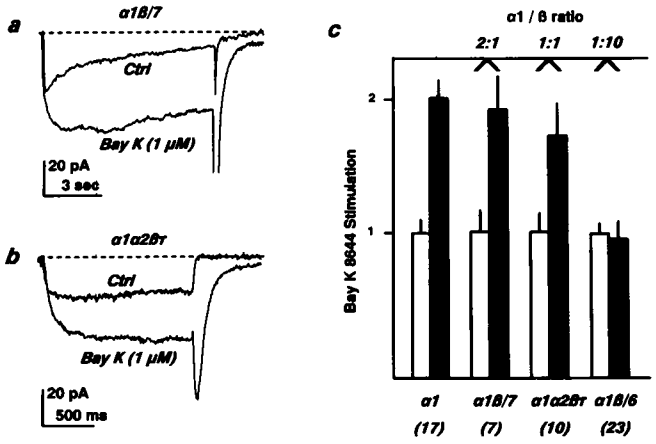

FIGURE 3 Effect of the SKMβ subunit on the Bay K 8644 modulation of Ba^{2+} currents. (*a*) Ba^{2+} current and its Bay K 8644 sensitivity (1 μM) recorded on a cell coexpressing SKMα1 and SKMβ (Lα1β/7 cell line), HP − 60 mV; TP + 20 mV. (*b*) The same experimental conditions for a cell coexpressing SKMα1, SKMα2, SKMβ, and SKMγ (Lα1α2$\beta\gamma$/3 cell line). (*c*) Ratio of the Ba^{2+} current stimulation by Bay K 8644 in cell lines coexpressing various ratios of SKMα1 and SKMβ. The open bars represent the normalized control current amplitudes. The filled bars stand for the Bay K 8644 stimulated current amplitudes. The mRNA ratio for SKMα_1 and SKMβ is presented on the top axis. The number of tested cells is given on the bottom axis.

of the skeletal L-type Ca channel occurs by a direct interaction of the β subunit with intracellular regions of the α1 protein. Such interaction is also consistent with previous biochemical studies (see reference 20 for review). Intracellular proteolytic treatment would result in a disruption of the complex. The simplest interpretation of these results is that the β subunit is removed by proteolytic treatment since α1 alone was not affected by such treatment. From the deduced amino acid sequences, the two subunits SKMα1 and SKMβ possess numerous putative tryptic cleavage sites (181 and 63, respectively). All of the intracellular regions of the SKMα1, i.e., intracellular connecting loops, the NH_2 terminal and the COOH terminal, exhibit such putative tryptic cleavage sites (a total of 112 sites). Interestingly, the loops between segments II and III and the COOH-terminal region contain the highest number of these trypsin recognition sites (24 and 55, respectively). The specific regions affected by the enzymatic treatment are not identified as yet. However, the primary structure of the β subunit displays regions (21), which are extremely sensitive to proteolysis (3). By analogy with the "ball and chain" model for the control of fast inactivation of Na^+ channels (22), we suggest the possibility that the β subunit may play a "ball" role on an intracellular "chain" of the complex. This model applies to the *Shaker* K^+ channel as well, in which the NH_2-terminal portion plays the role of the "ball" (15, 16). However, the regulation of Ca^{2+} channels may be more complex than the *Shaker* K^+ channel due to the multisubunit nature of the former. The ball may in fact be the SKMβ, an independent subunit. A similar situation might also occur in the case of Na^+ channels, for which inactivation could also be modulated by associated subunits (23–25). Such a role for the β subunit is critical for SKM Ca channel regulation since SKMα1, expressed alone, displays very slow gating (5–7).

The results also suggest that the structural determinants ascribed to DHP modulation are regulated from the intracellular side. The proteolysis experiments lend evidence that SKMβ can influence DHP sensitivity of SKM Ca^{2+} channels. In addition, the regulatory effects of the SKMβ on DHP modulation of SKMα1 depend on their relative level of expression, which is also consistent with a structural interaction between SKMα1 and SKMβ. The interaction between SKMα1 and SKMβ appears to be an essential feature for regulation and plasticity of skeletal muscle L-type Ca channels.

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REFERENCES

1. 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 (Lond.)*. 328:313–318.
2. Ellis, S. B., M. E. Williams, N. R. Ways, R. Brenner, A. H. Sharp, A. T. Leung, K. P. Campbell, E. McKenna, W. J. Koch, A. Hui, A. Schwartz, and M. M. Harpold. 1988. Sequence and expression of mRNAs encoding the α_1 and α_2 subunits of a DHP-sensitive calcium channel. *Science (Wash. DC)*. 241:1661–1664.
3. Ruth, P., A. Röhrkasten, M. Biel, E. Bosse, S. Regulla, H. E. Meyer, V. Flockerzi, and F. Hofmann. 1989. Primary structure of the β subunit of the DHP-sensitive calcium channel from skeletal muscle. *Science (Wash. DC)*. 245:1115–1118.
4. Jay, S. D., S. B. Ellis, A. F. McCue, M. E. Williams, T. S. Vedvick, M. Harpold, and K. P. Campbell. 1990. Primary structure of the γ subunit of the DHP-sensitive calcium channel skeletal muscle. *Science (Wash. DC)*. 248:490–492.
5. Perez-Reyes, E., H. S. Kim, A. E. Lacerda, W. Horne, W. Wei, D. Rampe, K. P. Campbell, A. M. Brown, and L. Birnbaumer. 1989. Induction of calcium currents by the expression of the α_1-subunit of the dihydropyridine receptor from skeletal muscle. *Nature (Lond.)*. 340:233–236.
6. Varadi, G., P. Lory, D. Schultz, M. Varadi, and A. Schwartz. 1991. Acceleration of activation and inactivation by the β subunit of the skeletal muscle calcium channel. *Nature (Lond.)*. 352:159–162.
7. Lacerda, A. E., S. K. Haeyoung, P. Ruth, E. Perez-Reyes, V. Flockerzi, F. Hofmann, L. Birnbaumer, and A. M. Brown. 1991. Nor-

malization of current kinetics by interaction between the α_1 and β subunits of the skeletal muscle dihydropyridine-sensitive Ca^{2+} channel. *Nature (Lond.).* 352:527–530.

8. Mikami, A., K. Imoto, T. Tanabe, T. Niidome, Y. Mori, H. Takeshima, S. Narumiya, and S. Numa. 1989. Primary structure and functional expression of the cardiac dihydropyridine-sensitive calcium channel. *Nature (Lond.).* 340:230–233.
9. Mori, Y., T. Friedrich, M-S. Kim, A. Mikami, J. Nakai, P. Ruth, E. Bosse, F. Hofmann, V. Flockerzi, T. Furuichi, K. Mikoshiba, K. Imoto, T. Tanabe, and S. Numa. 1991. Primary structure and functional expression from complementary DNA of a brain calcium channel. *Nature (Lond.).* 350:398–402.
10. Singer, D., M. Biel, I. Lotan, V. Flockerzi, F. Hofmann, and N. Dascal. 1991. The roles of the subunits in the function of the calcium channel. *Science (Wash. DC).* 253:1553–1557.
11. Wei, X., E. Perez-Reyes, A. E. Lacerda, G. Schuster, A. M. Brown, and L. Birnbaumer. 1991. Heterologous regulation of the cardiac Ca^{2+} channel α_1 subunit by skeletal muscle β and γ subunits. *J. Biol. Chem.* 266:21943–21947.
12. Klöckner, U., K. Itagaki, I. Bodi, and A. Schwartz. 1992. β-subunit expression is required for cAMP-dependent increase of cloned cardiac and vascular calcium channel currents. *Pfluegers Arch. Eur. J. Physiol.* 420:413–415.
13. Sambrook, F., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press Cold Spring Harbor, New York. 7.43–7.55.
14. Armstrong, C. M., F. Bezanilla, and E. Rojas. 1973. Destruction of sodium conductance inactivation in squid axons perfused with pronase. *J. Gen. Physiol.* 62:375–391.
15. Hoshi, T., W. N. Zagotta, and R. W. Aldrich. 1990. Biophysical and molecular mechanisms of *Shaker* potassium channel inactivation. *Science (Wash. DC).* 250:533–538.
16. Zagotta, W. N., T. Hoshi, and R. W. Aldrich. 1990. Restoration of inactivation in mutants of *Shaker* potassium channels by a peptide derived from *Sh.B. Science (Wash. DC).* 250:568–571.
17. Hescheler, J., and W. Trautwein. 1988. Modification of L-type calcium current by intracellularly applied trypsin in guinea-pig ventricular myocytes. *J. Physiol. (Lond.).* 404:259–274.
18. Klöckner, U. 1988. Isolated coronary smooth muscle cells: increase in the availability of L-type calcium channel current by intracellularly applied peptidases. *Pfluegers Arch. Eur. J. Physiol.* 412:R82. (Abstr.).
19. Obejero-Paz, C. A., S. W. Jones, and A. Scarpa. 1991. Calcium currents in the A7r5 smooth muscle-derived Cell Line. *J. Gen. Physiol.* 98:1127–1140.
20. Vaghy, P. L., E. McKenna, K. Itagaki, and A. Schwartz. 1988. Resolution of the identity of the calcium antagonist receptors in skeletal muscle. *Trends Pharmacol. Sci.* 9:398–402.
21. Rogers, S., R. Wells, and M. Rechsteiner. 1986. Amino acid sequences common to rapidly degraded proteins: The PEST hypothesis. *Science (Wash. DC).* 234:364–368.
22. Bezanilla, F., and C. M. Armstrong. 1977. Inactivation of the sodium channel II. Gating current experiments. *J. Gen. Physiol.* 70:567–590.
23. Auld, V. J., A. L. Goldin, D. S. Krafte, J. Marshall, J. M. Dunn, W. A. Catterall, H. A. Lester, N. Davidson, and R. J. Dunn. 1988. A rat brain Na channel α subunit with novel gating properties. *Neuron.* 1:449–461.
24. Krafte, D. S., A. L. Goldin, V. J. Auld, R. J. Dunn, N. Davidson, H. A. Lester. 1990. Inactivation of cloned Na channels expressed in *Xenopus* Oocytes. *J. Gen. Physiol.* 96:689–706.
25. Isom, L. L., K. S. DeJongh, D. E. Patton, B. F. X. Reber, J. Offord, H. Charbonneau, K. Walsh, A. Goldin, and W. A. Catterall. 1992. Primary structure and functional expression of the β_1 subunit of the rat brain sodium channel. *Science (Wash. DC).* 256:839–842.