Trypsin Increases Availability and Open Probability of Cardiac L-Type Ca^{2+} Channels Without Affecting Inactivation Induced by Ca^{2+}

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ABSTRACT The patch-clamp technique was employed to investigate the response of single L-type Ca^{2+} channels to the protease trypsin applied to the intracellular face of excised membrane patches from guinea pig ventricular myocytes. Calpastatin and ATP were used to prevent run-down of Ca^{2+} channel activity monitored with 96 mM Ba^{2+} as charge carrier in the presence of 2.5 μM (−)-BAYK 8644. Upon application of trypsin (100 μg/ml) channel activity was enhanced fourfold and remained elevated upon removal of trypsin, as expected of a proteolytic, irreversible modification. The trypsin effect was not mediated by a proteolytic activation of protein kinases, as evidenced by the insensitivity of this effect to protein kinase inhibitors. Trypsin-modified Ca^{2+} channels exhibited the usual run-down phanomenon upon removal of calpastatin and ATP. In ensemble average currents trypsin-induced changes of channel function are apparent as a threefold increase in peak current and a reduction in current inactivation. At the single channel level these effects were based on about a twofold increase in both Ca^{2+} channels' availability and open probability. Neither the actual number of channels in the patch nor their unitary conductance as well as reversal potential was changed by trypsin. The Ca^{2+}-induced inactivation was not impaired, as judged by a comparable sensitivity of trypsin-modified Ca^{2+} channels to intracellular Ca^{2+}. Similarly, trypsin treatment did not affect the sensitivity of Ca^{2+} channels to phenylalkylmine inhibition. The observed alterations in channel function are discussed in terms of possible structural correlates.

INTRODUCTION

Voltage-dependent Ca^{2+} channels serve as signal transducers in muscle as well as nerve cells. Depolarization leads to the opening of Ca^{2+} channels, which thereafter inactivate by voltage- and Ca^{2+}-dependent mechanisms (Eckert and Chad, 1984; Carbone and Swandulla, 1989; McDonald et al., 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). The α_1 subunits of muscle-type Ca^{2+} channels show large homology in their primary sequence, with the highest 93–95% homology between cardiac and smooth muscle α_1 (Biel et al., 1990; Koch et al., 1990). Whereas intramembrane domains serve as pore region and voltage sensor, several mechanisms of channel regulation such as phosphorylation, inactivation, drug binding and interaction with subunits apparently involve intracellular domains (Varadi et al., 1995). Hence, intracellular application of proteases (Armstrong et al., 1973) may allow us to directly observe how modification of accessible channel protein domains affects channel function.

Thus far, there have been two main whole-cell studies of protease effects on cardiac and smooth muscle L-type Ca^{2+} channels (Hescheler and Trautwein, 1988; Obejero-Paz et al., 1991). Both investigators reported an increase in peak inward Ca^{2+} currents and a decrease in current inactivation. In cardiac Ca^{2+} channels this decrease has been proposed to be due to a removal of Ca^{2+}-dependent inactivation (Hescheler and Trautwein, 1988), whereas in smooth muscle cells it has been related to an impairment of voltage-dependent rather than Ca^{2+}-dependent inactivation (Obejero-Paz et al., 1991).

In the present study we investigated the effect of the protease trypsin on cardiac Ca^{2+} channel activity stabilized in the excised inside-out patch. Alterations of channel function apparent in ensemble average currents were interpreted at the single-channel level. Furthermore, damage of specific intracellular protein domains was examined by testing for sensitivity of trypsin-modified Ca^{2+} channels to phenylalkylamines and intracellular Ca^{2+}.

MATERIALS AND METHODS

Materials

Calpastatin (P-0787) and trypsin (T-8128) were purchased from Sigma (Vienna, Austria). Calpastatin was dialyzed overnight versus bath solution (see below). Trypsin (10 mg/ml) was dissolved in aqua bidest. and stored at −25°C. H-7 (1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride, H-121) and KN-62 ((1-(*N,O*-bis-(5-Isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl)-4-phenylpiperazine) were supplied by Research Biochemical International (Vienna, Austria) and Calbiochem (Vienna, Austria), respectively. The phenylalkyamine derivatives D600 and D890 were kindly provided by Fa. Knoll (Ludwigshafen, Germany). Stock so-

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lutions (10 mM) of protein kinase inhibitors and D600 were prepared with dimethyl sulfoxide, D890 (100 mM) was dissolved in bidisti water.

Electrophysiological measurements

Single-channel tight-seal patch-clamp recordings (Hamil et al., 1981) were obtained from freshly isolated guinea pig ventricular myocytes (Romanin et al., 1991) with a List L/M EPC 7 amplifier. Soft glass patch pipettes had resistances between 1.4 and 2.0 MΩ after filling with a solution containing (mM): 0.0025 (−)-BAYK 8644, 96 $BaCl_2$, 5 HEPES/Na, pH 7.35. Bath solution consisted of (mM): 110 aspartic acid, 20 KCl, 2 EGTA, 2 $MgCl_2$, 20 HEPES/K, pH 7.4. In some experiments employing Li^+ instead of Ba^{2+} as the charge carrier pipette solution contained (mM): 0.0025 (−)-BAYK 8644, 140 LiCl, 5 EDTA, 5 HEPES/Li, pH 7.6. L-type Ca^{2+} channel activity was stabilized in the inside-out patch (Romanin et al., 1991; Seydl et al., 1995) with Sigma (2 U/ml) calpastatin and ATP/Na_2 (1 mM), which both were perfused into the bath chamber before patch excision. Inclusion of BAYK 8644 in pipette solutions enabled persistent Ca^{2+} channel stabilization for at least 20 min. Single-channel activities were recorded first in the cell-attached patch (c.a.), and then after patch excision in the inside-out (i.o.) configuration during repetitive (0.25 Hz) depolarizations for 1.45 s from a holding potential of −45 mV to 0 mV. Steady-state voltage-dependent inactivation was not observed at the usually applied holding potential of −45 mV, as its lowering to −80 mV did not result in a significant increase in Ca^{2+} channel activity. Current-to-voltage relationships were obtained either by depolarizing voltage steps from −30 mV to 40 mV at 10-mV intervals or by voltage ramps from −45 mV to 80 mV. With Li^+ as charge carrier the voltage protocol for Ca^{2+} channel activation was shifted by −40 mV, producing depolarizations from −85 mV to −40 mV (Hess et al., 1986). Single channel traces were filtered at 500 Hz (−3 dB 2-pole bessel filter) and digitized at 1.5 kHz.

Evaluation of trypsin modification of Ca^{2+} channel activity was primarily based on the time course of average channel activity ($N \cdot p$) determined for each depolarizing voltage pulse (Seydl et al., 1995). Furthermore, ensemble average currents (average of usually 10–50 current traces) were used to evaluate trypsin effects on peak inward current (I_{peak}) and inactivation. Inactivation (in percent) was calculated by $[100 \times (1 - I_{1450}/I_{peak})]$ with I_{1450} as the ensemble average current measured 1450 ms after the onset of depolarization. Modification of single-channel characteristics was judged by unitary current-to-voltage relationships, channel availability (P_s), and open probability (p). P_s was defined by the ratio (×100 in percent) of depolarizing sweeps with channel activity to the total number of sweeps, and p represents the channel's open probability in non-blank sweeps. To allow estimation of these parameters also from experiments with more than one single channel, a procedure was developed enabling determination of the number of channels present in the patch (n), their P_s, and p (see Appendix). Calculation of ensemble average currents and of all point amplitude histograms were performed employing pClamp 5.7.2 and 6.0.2 software of Axon Instruments. Average currents were constructed from capacity- and leak-corrected single-channel traces by subtraction of traces showing no channel openings. Thus, the baseline is identical to the zero current level indicated by the dashed line. Mean ± SD values are presented throughout the paper.

RESULTS

In an attempt to study the effect of the protease trypsin on L-type Ca^{2+} channels in the inside-out patch, run-down of channel activity, which is usually observed upon patch excision (Cavalie et al., 1983), was prevented by calpastatin and ATP (Romanin et al., 1991; Seydl et al., 1995). Ca^{2+} channel activity was monitored with Ba^{2+} as charge carrier to predominantly observe voltage-dependent inactivation during long depolarizations (1.45 s) to 0 mV (Yue et al., 1990a; Giannattasio et al., 1991). Application of trypsin (100 μg/ml) to the intracellular face of the membrane patch induced a profound effect on L-type Ca^{2+} channel currents, as exemplified in Fig. 1. About a fivefold increase in channel activity (determined by $N \cdot p$) occurred within 3 min after trypsin application and was followed by a slight decrease in its continuous presence (Fig. 1 *A*). Washout of trypsin as well as calpastatin plus ATP resulted in a complete run-down of trypsin-modified Ca^{2+} channel activity, as similarly observed with native Ca^{2+} channels (Romanin et al., 1991). Ensemble average currents as shown in Fig. 1 *B* were then used for a further characterization of the observed increase in $N \cdot p$. Whereas channel activity in the cell-

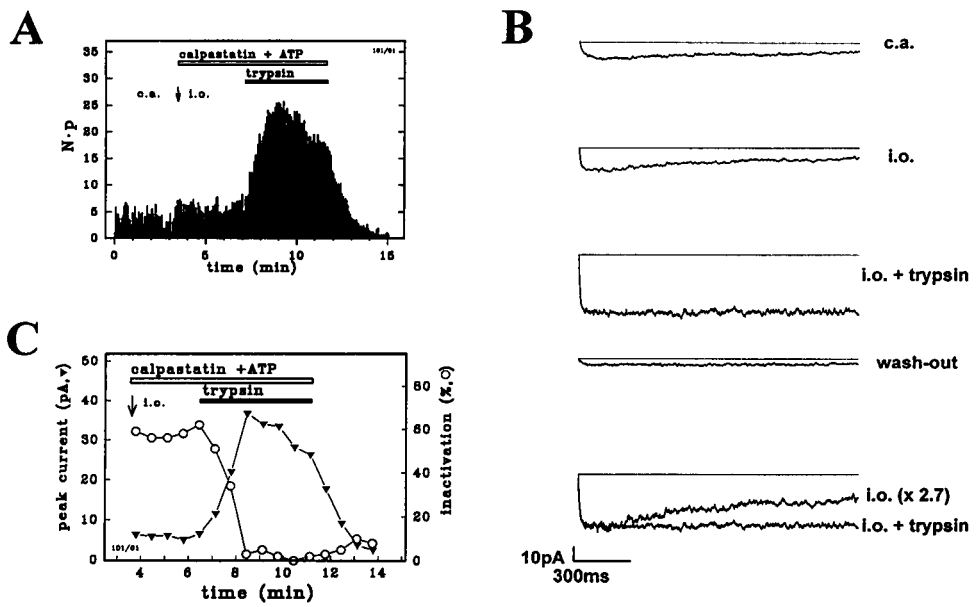

FIGURE 1 Increase in cardiac Ca^{2+} channel activity by trypsin. (*A*) Time course of Ca^{2+} channel activity determined as N·p in the cell-attached (c.a.) and inside-out (i.o.) patch followed by application of trypsin (100 μg/ml) to the intracellular face of the membrane patch. At 11.8 min washout of calpastatin + ATP and trypsin. (*B*) Ensemble average currents determined under the various experimental conditions as indicated. (*C*) Time course of peak amplitude and inactivation estimated from ensemble average currents. All data are from one experiment.

attached patch was quite similar to that after patch excision (compare c.a and i.o. trace in Fig. 1 *B*), the augmentation of Ca^{2+} channel activity induced upon trypsin was caused by two effects, as clearly evident from the third trace (i.o. + trypsin) in Fig. 1 *B*, i.e., an increase in peak average current and an attenuation of inactivation. The latter effect is obvious from the lowest panel of Fig. 1 *B*, where control average current in the inside-out patch was scaled up by a factor of 2.7 to match peak average current after trypsin treatment. The time course of trypsin effect on both peak inward current and inactivation (Fig. 1 *C*) shows that a change in these parameters occurred initially in parallel at our time resolution of about 0.7 min. In the prolonged presence of trypsin we observed a moderate decrease in peak average current, which was dramatically speeded up upon washout of calpastatin, ATP, and trypsin. In contrast, inactivation remained quite constant during this run-down of channel activity (Fig. 1 *B*, fourth trace, and Fig. 1 *C*). Thus, activity of trypsin-modified Ca^{2+} channels in the inside-out patch (see below Fig. 3 *A*) is still dependent on the presence of calpastatin plus ATP.

Statistics of trypsin effects on Ca^{2+} channel activity

Fig. 2 presents an overview of trypsin effects evaluated from ensemble average currents. In 28 of 32 experiments performed as shown in Fig. 1, trypsin produced a significant increase in average Ca^{2+} channel activity. Among these 28 experiments five yielded an average current that was too noisy to allow for an accurate measurement of peak current as well as inactivation. Three experiments were impaired by an overlaid run-down of channel activity, and two experiments showed almost no inactivation of average currents already in the inside-out patch. Thus, 18 experiments were further analyzed. After trypsin application the maximal effect was observed on average within 3.4 min (earliest, 1 min; latest, 10 min). The logarithmic plot in Fig. 2 *A* demonstrates how consistently trypsin produced an increase in $N \cdot p$. Comparison of Ca^{2+} channel activity before and after trypsin revealed a fourfold augmentation of $N \cdot p$ (n = 18). The trypsin-induced increase in peak average current could be fitted by a linear correlation with a slope of about 3 (Fig. 2 *B*). A significantly positive current at the intersection of the fitted line with the ordinate might indicate recruitment of previously silent channels upon trypsin treatment. However, calculation of 95% confidence limits revealed no significance for a fit delineating from zero. Trypsin consistently produced a reduction in inactivation from 50 ± 16% before to 17 ± 11% (n = 18) after its application (Fig. 2 *C*). Inactivation of control Ca^{2+} channels showed large variations from about 20% to 70%, in accordance with previous reports (Cavalie et al., 1986; Haack and Rosenberg, 1994). The amount of inactivation was not positively correlated with the magnitude of peak average current, as shown in Fig. 2 *D* to be consistent with voltage- rather than

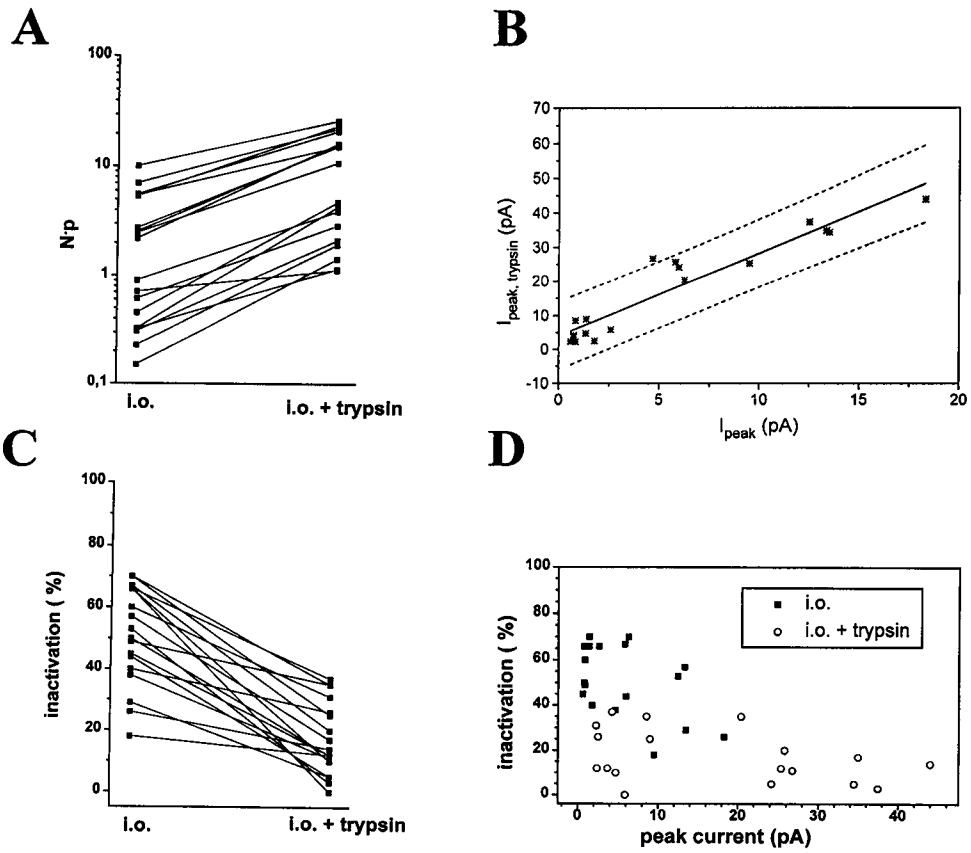

FIGURE 2 Statistics of trypsin effects. (*A*) Increase in Ca^{2+} channel activity ($N \cdot p$) after trypsin application. (*B*) Correlation of peak average current before (I_{peak}) and after trypsin (100 μg/ml) application ($I_{peak, trypsin}$). Dashed lines represent 95% confidence limits for the continuous line fitted by linear regression. (*C*) Reduction of average current inactivation following trypsin application. (*D*) Peak amplitudes are plotted versus inactivation each determined from ensemble average currents before and after trypsin yielding correlation coefficients of −0.45 and −0.39, respectively.

current (Ba^{2+})-dependent inactivation (Mazzanti et al., 1991). Accordingly, substituting Li^+ as charge carrier for Ba^{2+} to completely eliminate divalent-induced Ca^{2+} channel inactivation (Hadley and Hume, 1987) trypsin application resulted in a similar 46% reduction of inactivation concomitant to about a fivefold increase in peak average currents (data not shown).

In summary, trypsin increased cardiac Ca^{2+} channel activity, which results in enhancement of peak inward current concomitant to a reduction of inactivation. Because the macroscopic current (I) is related to microscopic parameters by $I = nP_s ip$ (Klöckner and Isenberg, 1994), where n, P_s, i, and p refer to the number of channels in the patch, their availability, the single-channel amplitude, and the open probability, respectively, an increase in I might be caused by an increase in any of these parameters. In the following, microscopic parameters were determined to further characterize the basis of the aforementioned trypsin effects.

Trypsin does not change the permeability characteristics of single Ca^{2+} channels

Fig. 3 shows a typical trypsin experiment with a resolution of single Ca^{2+} channels enabling a comparison of unitary conductance and reversal potential before and after trypsin application. The expected irreversibility of trypsin effect was confirmed here, as evident from the time course in Fig. 3 *A* showing persistance of enhanced channel activity (N·p) after trypsin removal. Comparison of single-channel amplitudes determined from amplitude histograms (Fig. 3 *C*) of respective single-channel traces (exemplified in Fig. 3 *B*) did not reveal a significant change after trypsin treatment. Furthermore, neither single-channel conductance (25.5 pS in control and 25.3 pS in trypsin-modified channels) nor reversal potential (in both cases, 63 mV) was altered. Thus, the trypsin-induced augmentation of Ca^{2+} channel activity is apparently caused by alterations of n, P_s and p. In the following, P_s was defined by the ratio (as a percentage) of

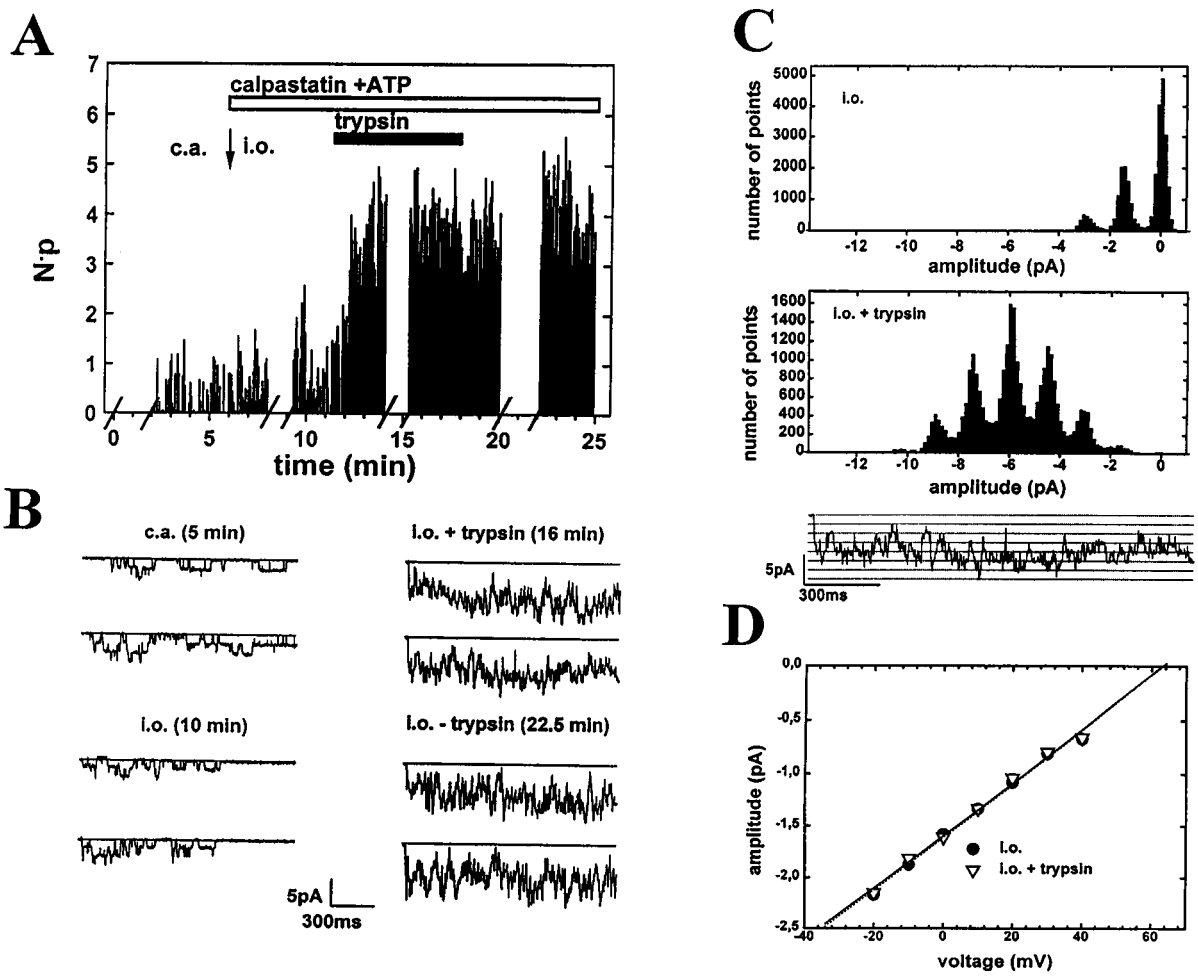

FIGURE 3 Trypsin did not affect Ca^{2+} channel permeation. (*A*) Time course of Ca^{2+} channel activity (N·p) in the cell-attached (c.a.) and inside-out (i.o.) patch. Trypsin (100 μg/ml) was applied between 11.1 min and 18.0 min. In the time periods showing no activity unitary current-voltage relationships were recorded. (*B*) Corresponding single channel traces recorded consecutively at the indicated times. (*C*) Amplitude histograms constructed from 10–15 traces. Below histograms one single channel trace of *B* (i.o. + trypsin, lower trace) is shown with an expanded time scale and seven open channel levels indicated by dashed lines. (*D*) Unitary current-voltage relationships determined before and after trypsin application. All data are from one experiment.

depolarizing sweeps with channel activity to the total number of sweeps (Ochi and Kawashima, 1990), and p was calculated for non-blank sweeps.

Trypsin increases Ca^{2+} channel availability and open probability

An analysis method (see Appendix) was developed enabling refined estimation of P_s and p from membrane patches with n ($n < 9$) channels. Fig. 4 summarizes the results as to how trypsin affected microscopic parameters n, P_s, and p. Although experiments with one single Ca^{2+} channel were extremely difficult to perform because of a lower efficency of calpastatin plus ATP in channel stabilization, we succeeded in three experiments to largely prevent the run-down of channel activity over a time scale of about 25 min. The time course of the trypsin effect on one single Ca^{2+} channel (Fig. 4 *A*) and corresponding single channel traces (Fig. 4 *B*) revealed an increase in both P_s and p. However, no significant change in the number of channels as estimated in these three single-channel and in further three multi-channel experiments indicated that trypsin might not recruit previously silent channels. Comparison of P_s and p calculated for the inside-out patch before and after trypsin application revealed an increase from 42 ± 9% to 89 ± 10% and from 19 ± 4% to 44 ± 13%, respectively (Fig. 4 *C*). For the cell-attached patch similar values of P_s (47 ± 3%) and p (19 ± 10%) were calculated as for the inside-out patch, substantiating the efficiency of calpastatin plus ATP in stabilizing Ca^{2+} channel activity after patch excision.

In conclusion, an equal (twofold) increase in both channel availability and open probability apparently accounts for the

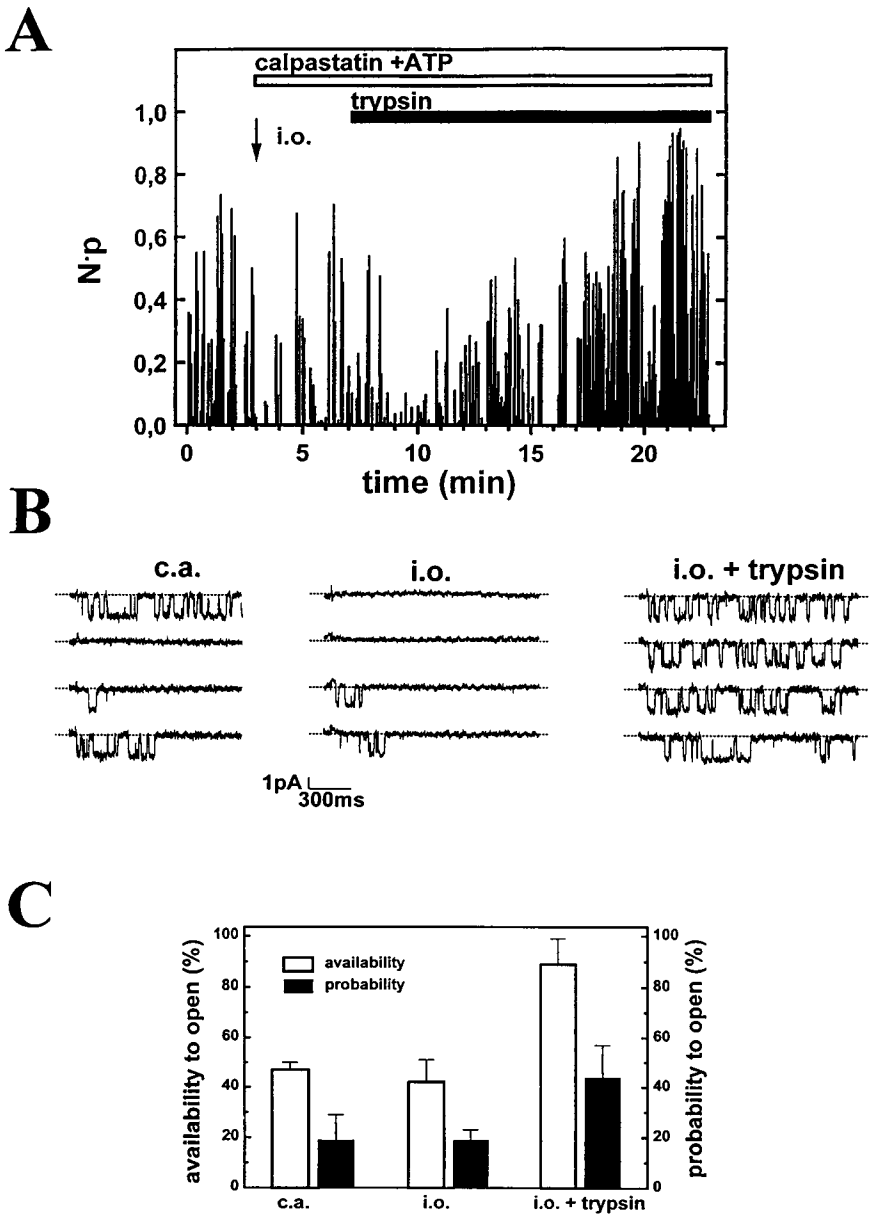

FIGURE 4 Trypsin increased Ca^{2+} channel activity by increasing the channel's availability and open probability. (*A*) Time course of Ca^{2+} channel activty (N·p) as shown in Fig. 1 *A* with one single Ca^{2+} channel in the patch. (*B*) Corresponding single channel traces recorded consecutively at 1 min (c.a.), 3.3 min (i.o.), and 17.1 min (i.o. + trypsin). (*C*) Availability and open probability (within non-blank sweeps) determined in the cell-attached (c.a.) and inside-out (i.o.) patch before and after trypsin. Results are from six experiments.

observed fourfold enhancement of Ca^{2+} channel activity ($N \cdot p$) determined from ensemble average currents.

Protein kinases are not involved in trypsin effects on Ca^{2+} channels

An increase in P_s and p of the cardiac Ca^{2+} channel activity is also known to occur in response to stimulation of PK-A activity (Ochi and Kawashima, 1990; Yue at al., 1990b; Hirano et al., 1994). As trypsin has been reported to activate adenylate cyclase (Cros et al., 1985) and consequently protein kinase A, the protein kinase inhibitor H7 (Inagaki et al., 1984; Kawamoto and Hidaka, 1984) was used to resolve an involvement of protein kinases in the observed trypsin-induced modifications of Ca^{2+} channel activity. Fig. 5 shows a typical experiment where H7 (20 μM) was applied after Ca^{2+} channel stabilization and was continuously present during trypsin application. Neither the time course of $N \cdot p$ (Fig. 5 *A*) nor ensemble average currents (Fig. 5 *B*) exhibited an effect of H7 on control as well as trypsin-modified Ca^{2+} channel activity. Similarly, the activation maximum of channel activity determined from current responses to voltage ramps was not altered by trypsin (Fig. 5 *C*). In three experiments protein kinase inhibition by 20–100 μM H7 did not significantly affect stabilized Ca^{2+} channel activity, which was, however, enhanced upon trypsin application by a factor of 3.9 ± 1.9. Similar results were obtained in the additional presence of KN-62 (Tokumitsu et al., 1990), an inhibitor of Ca^{2+}/calmodulin-dependent kinase (data not shown). Thus, protein kinases are apparently not involved in the trypsin-induced enhancement of Ca^{2+} channel activity. L-type Ca^{2+} channel activity was here additionally confirmed by its complete inhibition upon application of the dihydropyridine (±) PN 200 110 (20 μM).

Ca^{2+}-induced inactivation is not impaired in trypsin-modified Ca^{2+} channels

Intracellular perfusion of trypsin in whole cell experiments has been reported to impair the Ca^{2+}-induced inactivation process of cardiac Ca^{2+} currents (Hescheler and Trautwein, 1988). Use of Ca^{2+} as charge carrier instead of Ba^{2+} to monitor Ca^{2+} channel activity was unfortunately not applicable, because under this condition Ca^{2+} channel activity could not be sufficiently stabilized in the inside-out patch. Therefore, in an attempt to resolve the efficacy of intracellular Ca^{2+} to promote inactivation of trypsin-modified Ca^{2+} channels, we used a recently established experimental approach (Romanin et al., 1992), monitoring the effect of defined intracellular Ca^{2+} concentrations on Ba^{2+} currents through Ca^{2+} channels. Fig. 6 presents the results of such a typical experiment. Specifically, Ca^{2+} channel activity was first subjected for control to an increase in the intracellular Ca^{2+} concentration from about 20 nM to 15 μM. Then, after lowering Ca^{2+} again to 20 nM, trypsin was applied to augment Ca^{2+} channel activity, which was subsequently reexposed to 15 μM Ca^{2+}. The time course of $N \cdot p$ is depicted in Fig. 6 *A*. It is clearly evident that control as well as trypsin-modified Ca^{2+} channel activity was sensitive to the elevation of intracellular Ca^{2+} to 15 μM. Ca^{2+} channel activity ($N \cdot p$) of control and of trypsin-modified channels was reduced to 10 ± 4% (n = 3) and 15 ± 11% (n = 5), respectively, upon exposure to 15 μM intracellular Ca^{2+}. Respective ensemble average currents as exemplified in

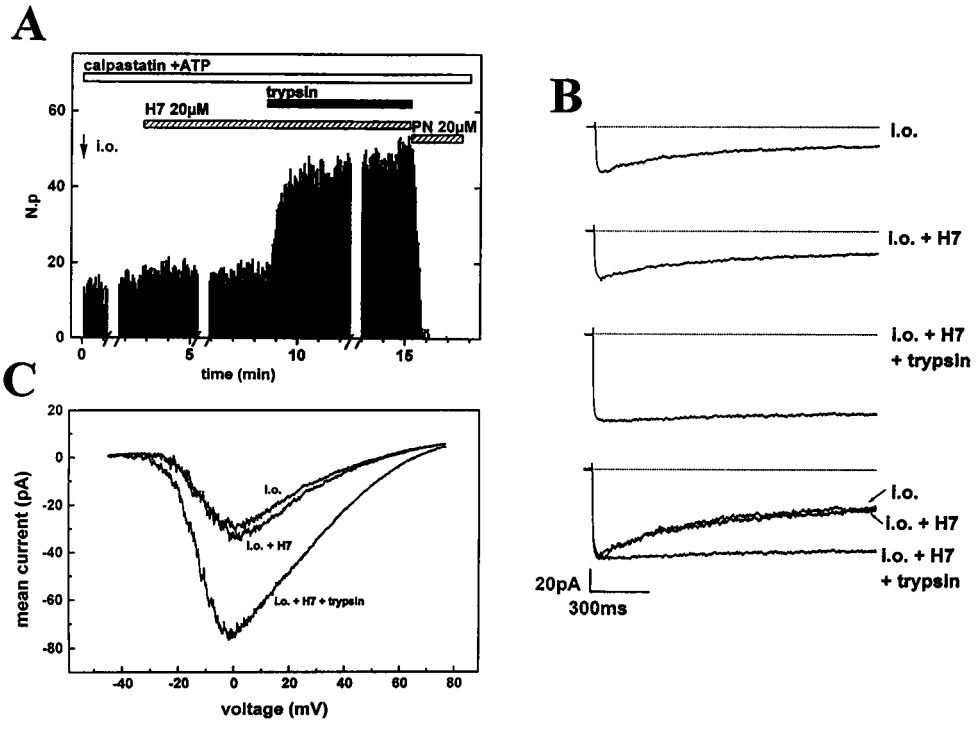

FIGURE 5 Protein kinase activity is not involved in the trypsin-induced increase in Ca^{2+} channel activity. (*A*) Time course of Ca^{2+} channel activity ($N \cdot p$) in the inside-out (i.o.) patch followed by application of the protein kinase inhibitor H7 (20 μM) and trypsin (100 μg/ml). Ca^{2+} channel activity was finally completely blocked by (±) PN 200 110 (20 μM). (*B*) Ensemble average currents determined under the various experimental conditions as indicated. (*C*) Mean current responses to slow (2.44 s) voltage ramps from −45 mV to 80 mV recorded under the indicated conditions.

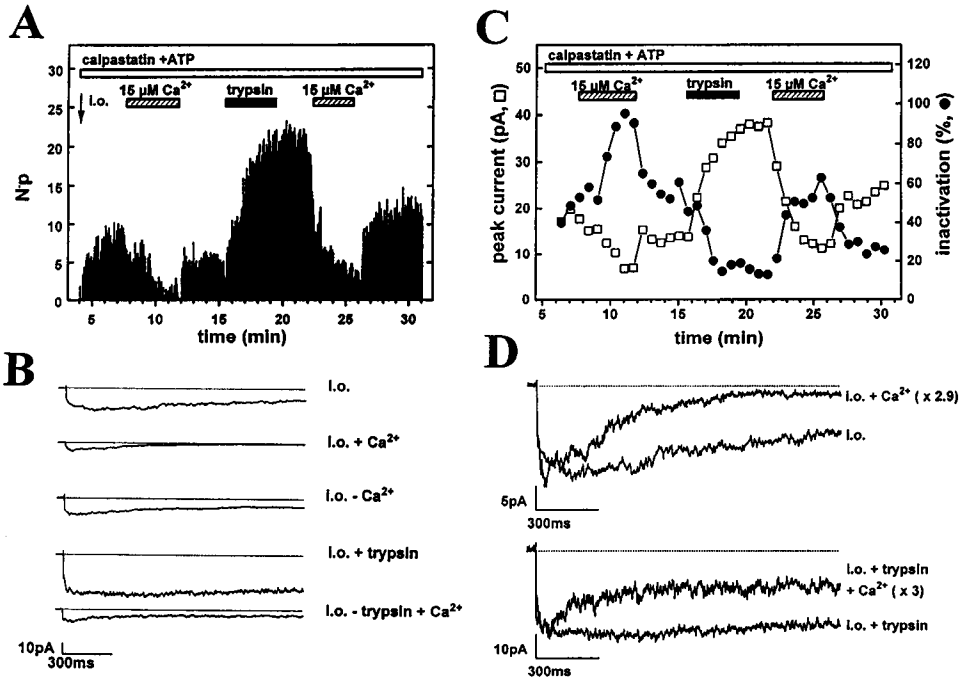

FIGURE 6 Trypsin did not impair Ca^{2+}-induced inactivation of Ca^{2+} channels. (*A*) Time course of Ca^{2+} channel activity (N·p) in the inside-out (i.o.) patch exposed to intracellular Ca^{2+} concentrations from about 20 nM to 15 μM before and after trypsin application. (*B*) Ensemble average currents determined under the various experimental conditions as indicated. (*C*) Time course of peak amplitude and inactivation of ensemble average currents. (*D*) Average currents scaled up by the indicated factors to match peak current size. All data are from one experiment.

Fig. 6 *B* indicate an effect of Ca^{2+} elevation on both peak inward current and inactivation as previously reported (Romanin et al., 1992). Both parameters were affected on a similar time scale and to a similar extent in control as well as trypsin-modified Ca^{2+} channels (Fig. 5 *C*). Inactivation as well as peak inward current were reduced by a comparable amount before and after trypsin treatment (Fig. 5 *D*). Specifically, elevation of intracellular Ca^{2+} to 15 μM inhibited peak inward Ba^{2+} current to 33 ± 22% (n = 3) before and after trypsin application to 23 ± 13% (n = 5). Inactivation amounted to 49 ± 4% (n = 3) in control and 18 ± 10% (n = 5) in trypsin-modified channel activity and was enhanced in the presence of 15 μM Ca^{2+} to 88 ± 7% (n = 3) before and 50 ± 23% (n = 5) after trypsin application. Thus, we conclude that trypsin apparently did not impair Ca^{2+}-dependent inactivation.

Trypsin-modified Ca^{2+} channels are still sensitive to phenylalkylamine inhibition

As the binding domain of phenylalkylamines is located intracellularly at the carboxy tail close to the VIS6 of the α_1-subunit (Catterall and Striessnig; 1992), we examined for a possible impairment of phenylalkylamine inhibition upon trypsin treatment using the permanently charged compound D890. Fig. 7 depicts two kinds of experiments which revealed that inhibition of Ca^{2+} channel activity by D890 occurred independently of the trypsin effect. The dose-dependent inhibition of Ca^{2+} channel activity by D890 (Fig. 7 *A*) was followed by the typical increase upon trypsin application. Consistently, trypsin-modified Ca^{2+} channel activity was sensitive to inhibition by D890 (Fig. 7 *B*). Qualitatively similar results were obtained with the phenylalkylamine D600 (data not shown). Hence, the intracellular binding domain for phenylalkylamines is apparently not impaired by trypsin.

DISCUSSION

The major results presented here are that trypsin enhanced single cardiac Ca^{2+} channel activity by increasing the channel's availability and open probability without modifying the number of functional channels in the patch. Ion permeation was apparently not affected, as judged by a lack of trypsin effect on unitary conductance and reversal potential. In ensemble average Ba^{2+} currents trypsin-induced changes are apparent as an increase in peak amplitude and a reduction of inactivation. Hence, voltage-dependent inactivation is presumably impaired in trypsin-modified Ca^{2+} channels, whereas inactivation induced by intracellular Ca^{2+} was still present. Similarly, channel activity remained sensitive to phenylalkylamine and dihydropyridine inhibition.

Trypsin effects on cardiac and smooth muscle L-type Ca^{2+} channels

In a whole cell study on cardiac myocytes Hescheler and Trautwein (1988) report about a threefold increase in peak Ca^{2+} inward currents followed by a reduction in current inactivation upon intracellular trypsin application. When Ba^{2+} is used as the charge carrier instead of Ca^{2+}, the amplitude-enhancing effect is equally apparent, whereas inactivation is not diminished (Hescheler and Trautwein, 1988). This observation has led the above authors to suggest that trypsin impairs the Ca^{2+}-induced inactivation process.

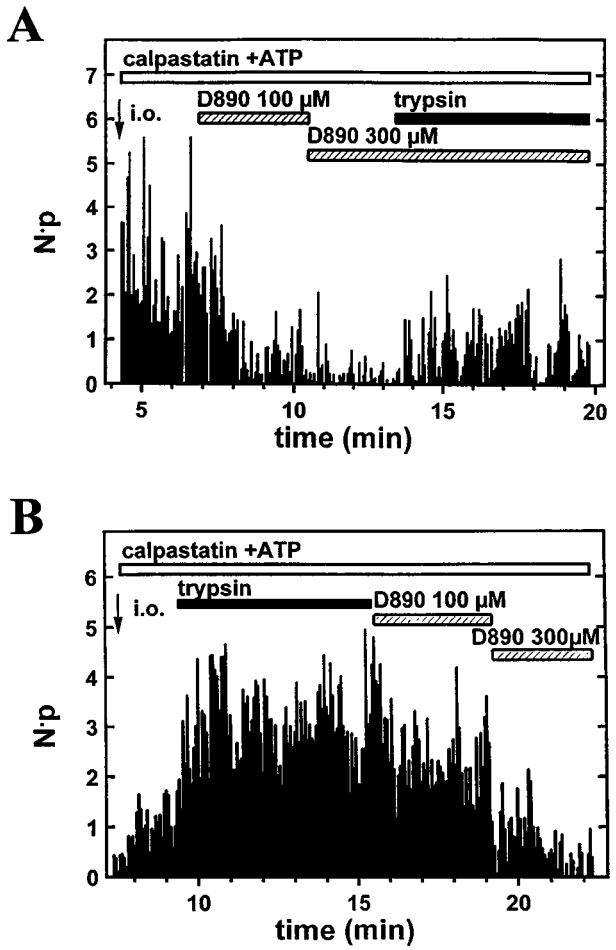

FIGURE 7 Trypsin-modified Ca^{2+} channels are sensitive to inhibition by D890. (*A*) Time course of Ca^{2+} channel activity (N·p) in the inside-out (i.o.) patch exposed to D890 (100, 300 μM) and additionally to trypsin (100 μg/ml). (*B*) Time course of Ca^{2+} channel activity (N·p) in the inside-out patch exposed first to trypsin (100 μg/ml) and subsequently to D890 (100, 300 μM).

In the present study trypsin produced about a threefold increase in peak ensemble average Ba^{2+} currents, perfectly matching the aforementioned results. However, concomitant to this increase a clear reduction of inactivation of Ba^{2+} average currents was also observed. The latter finding contrasts with a trypsin effect on inactivation of Ca^{2+} but not Ba^{2+} whole cell currents, as reported by Hescheler and Trautwein (1988). One reason for these divergent results might be found in the inside-out patch configuration employed in the present study, where components possibly affecting channel function would be lost upon patch excision. Alternatively, one may speculate that inactivation of Ba^{2+} average currents as observed in this study might in part represent current-dependent inactivation (Mazzanti et al., 1991). However, such current-dependent inactivation is rather unlikely, because no positive correlation was found between the peak of ensemble average Ba^{2+} currents and their inactivation. Furthermore, trypsin-modified Ca^{2+} channels still responded to an increase in intracellular Ca^{2+} by showing inactivation. As trypsin produced a similar reduction of inactivation with Li^{+} as charge carrier, we thus suggest that reduction of Ba^{2+} current inactivation by trypsin is apparently due to the proteolytic cleavage of intracellular domains responsible for voltage-dependent inactivation. Partially consistent results have been very recently reported in abstract form (Klöckner at al., 1995), where intracellular trypsin perfusion of transfected HEK 293 cells expressing cardiac α_1 and β_3 subunits together with skeletal muscle α_2 has been found to affect whole-cell Ba^{2+} currents by about a threefold increase in peak amplitude, with a minor effect on inactivation.

In a whole cell study (Obejero-Paz et al., 1991) on smooth muscle-type cells (line A7r5), trypsin infusion has been reported to increase peak Ba^{2+} currents and to selectively remove voltage-dependent inactivation, while preserving the more rapid Ca^{2+}-dependent inactivation. The latter study has further employed ensemble analysis to suggest that the main effect of trypsin is to increase the number of functional channels possibly with a smaller effect on their open probability. The results obtained here for single cardiac Ca^{2+} channels are in clear accordance with those proposed above for smooth muscle Ca^{2+} channels. In addition, an abstract by Klöckner (1988) reports that in coronary smooth muscle cells trypsin produces a fourfold increase in peak amplitude of whole cell Ca^{2+} currents with no effect on inactivation kinetics. Further analysis of single Ca^{2+} channel currents from cell-attached patches with Ba^{2+} as the charge carrier has assigned the trypsin effect to a significant increase in channels' availability, with a moderate effect on their open probability.

In conclusion, cardiac and smooth muscle Ca^{2+} channels apparently show a qualitatively similar response to trypsin consistent with the 95% homology between the channel types. Particularly, intracellular domains of both channels are identical, except for small portions of the amino and carboxyl termini as well as an insertion within the linker between domain I and II in smooth muscle α_1 (Biel et al., 1990; Koch et al., 1990).

As phosphorylation/dephosphorylation processes were apparently not involved in the stabilization as well as trypsin-induced stimulation of Ca^{2+} channel activity, a direct effect of trypsin on the cardiac Ca^{2+} channel protein appears very likely.

Possible structures involved in the increase of Ca^{2+} channel availability

As most of the basic characteristics of Ca^{2+} channel function are found when the α_1 subunit is expressed alone, its intracellular domains are considered first as targets for trypsin. Because trypsin is able to cleave at the amino acids arginine and lysin, about 160 putatively intracellular cleavage sites are present in the cardiac α_1-subunit. Cleavage at the large carboxyl tail, which possesses 78 putative sites for trypsin, might produce effects on channel function similar

to those found in those mutants where various amounts of the carboxyl terminus have been deleted (Wei et al., 1994). Omittance of 46% to 70% of the carboxyl tail of cardiac α_1-subunits expressed in oocytes has been reported to result in a three- to sixfold increase in peak Ba^{2+} currents (Wei et al., 1994). In a recent abstract, Klöckner et al. (1995) reported that carboxy-terminal deletion mutants (deletion of amino acids >1633), in contrast to the wild-type form of the cardiac α_1-subunit, no longer respond to trypsin application by an increase in peak Ba^{2+} currents. Thus, we suggest that part of the carboxyl terminus is at least involved in the regulation of cardiac Ca^{2+} channel availability. Interestingly, despite the proteolytic and thus irreversible enhancement of Ca^{2+} channel currents, channel activation was still dependent on the presence of calpastatin and ATP. Hence, proteolytically accessible intracellular domains of the Ca^{2+} channel protein are apparently not involved in the stabilization of Ca^{2+} channel activity.

Possible structures involved in voltage- and Ca^{2+}-dependent inactivation

Trypsin was found here to impair voltage-dependent inactivation, whereas Ca^{2+}-induced inactivation was obviously not affected in trypsin-modified Ca^{2+} channels. Thus, distinct sites are apparently responsible for these different types of inactivation of the cardiac Ca^{2+} channel consistent with the involvement of separate and independent mechanisms (Hadley and Lederer, 1991). Voltage-dependent inactivation as estimated from the inactivation rate of Ba^{2+} currents during depolarizing voltage pulses has been found (Zong et al., 1994) to occur to a similar extent upon expression of either cardiac wild-type or carboxy-terminal deletion mutant Ca^{2+} channels. Hence, trypsin modification of the carboxyl terminus alone is apparently not sufficient to affect voltage-dependent inactivation.

Ca^{2+} binding sites have been located to an intracellular EF hand motif close to IVS6 (Babitch, 1990; deLeon et al., 1995) or in a cluster of negatively charged amino acids in the loop connecting domains II and III of the α_1 subunit (Tanabe et al., 1987), which when expressed alone is sufficient to produce Ca^{2+}-dependent inactivation (Neely et al., 1994). As trypsin-modified Ca^{2+} channels were still sensitive to an elevation in intracellular Ca^{2+}, this putative Ca^{2+} binding motif is apparently not accessible to trypsin and might be located close to the membrane. The idea of a role of the EF hand motif is in line with this result and is further supported by the resistance of the adjacent phenylalkylamine binding domain (Catterall and Striessnig, 1992) to proteolysis by trypsin.

The results obtained here demonstrate a role of proteolytically accessible intracellular domains of the Ca^{2+} channel protein as determinants of channel availability and voltage-dependent inactivation. This may provide a basis for further studies with mutant Ca^{2+} channels to identify the protein structures responsible for the observed changes in channel function.

APPENDIX

Determination of the number of functional channels in a patch, their availability, and open probability

The availability (P_s) of the Ca^{2+} channel is defined as the chance to evoke channel activity upon a depolarizing voltage pulse, i.e., the ratio of sweeps exhibiting channel activity to all sweeps. In experiments with one single Ca^{2+} channel ($n = 1$), P_S and the open probability (p) can be easily determined, whereas in multi-channel experiments these parameters are more difficult to calculate. Therefore, a method was developed to allow for an estimation of the number of channels n, P_s, and p from channel traces with $n < 9$ channels, idealized using the L-filter algorithm (Pastushenko and Schindler, submitted for publication). The following assumptions are initially made:

i) The channels are identical and behave independently.

ii) The number of channels available in a sweep remains constant within the duration of the sweep.

Thus, one can assume that the number of active channels (n_A) obeys a binomial distribution with n as the number of channels in the patch and P_s as their availability. Then, the probability $p(n_A)$ of finding n_A channels available is described by

$$p(n_A) = \binom{n}{n_A} \cdot P_s^{n_A} \cdot (1 - P_s)^{n - n_A} \quad (1)$$

If n and the fraction of channels available to be opened in a sweep would be known, the availability could be estimated by

$$P_s \approx \frac{\sum_{n_A=0}^{n} m(n_A) \cdot n_A}{n \cdot M} \quad (2)$$

where $m(n_A)$ is the number of sweeps with n_A channels available and M is the total number of sweeps, which equals

$$M = \sum_{n_A=0}^{n} m(n_A) \quad (3)$$

n and n_A for each sweep could be reasonably calculated with a maximum estimator (Horn, 1991) if n is small and p large. This estimator is biased. Hence, a small p (<30%) and $n > 3$ might largely affect the result, because the probability that all available channels would open concomitantly in a sweep is low. In addition, a small availability (<30%) might lead to errors in estimating n.

Therefore, a maximum likelihood estimator was set up for determination of n, P_s and p, based on the following additional assumption.

iii) The process regulating channel gating within a sweep is with respect to the number of sampling points in each conductance level distribution-ergodic. Hence, the probabability of finding a sampling point in the kth conductance level can be described by a binomial distribution with the parameters n_A and p.

$$p(k) = \binom{n_A}{k} \cdot p^k \cdot (1 - p)^{n_A - k} \quad (4)$$

Equation (4) and assumption iii) allow us then to determine the probability for one sweep (Eq. 5) at given n_A with the following variables defined as

n	maximum number of channels in a patch
n_{Ai}	available channels in the ith sweep
t_{ik}	number of sampling points in the kth conductance level in the ith sweep
$\mathbf{T}_i$	vector of t_{ik} for the ith sweep
$\mathbf{T}$	matrix of all t_{ik}
T_{ges}	number of sampling points in a sweep
P_S	availability

p open probability
M total number of sweeps
K_i set of conductance level that occur in the ith sweep

The elements of $\mathbf{T}_i$ that describe the ith sweep obey a multinomial distribution (Weiß, 1987), and the probability of $\mathbf{T}_i$ at a given n_{Ai} and p is

$$p(\mathbf{T}_i|n_{Ai}, p) = \left\{\prod_{k=0}^{n_{Ai}}\left[\binom{n_{Ai}}{k} \cdot p^k \cdot (1-p)^{n_{Ai}-k}\right]^{t_{ik}}\right\} \frac{T_{ges}!}{\prod_{k\in K_i} t_{ik}!} \tag{5}$$

if n_{Ai} is higher than or equal to the highest conductance level in $\mathbf{T}_i$. If n_{Ai} is smaller than the highest conductance level, then

$$p(\mathbf{T}_i|n_{Ai}, p) = 0 \tag{6}$$

Because of the binomial distribution of n_{Ai} (Eq. 1) the probability of $\mathbf{T}_i$ at given n, P_S, and p is

$$p(\mathbf{T}_i|n, P_s, p) = \sum_{n_{Ai}=0}^{n} p(\mathbf{T}_i|n_{Ai}, p) \cdot \binom{n}{n_{Ai}} \cdot P_S^{n_{Ai}} \cdot (1-P_S)^{n-n_{Ai}} \tag{7}$$

leading to the probability of $\mathbf{T}$ comprising all sweeps

$$p(\mathbf{T}|n, P_S, p) = \prod_{i=1}^{M} p(\mathbf{T}_i|n, P_S, p) \tag{8}$$

Using Eq. (8), a maximum likelihood estimator for n, P_s, and p was constructed by maximizing the probability of $\mathbf{T}$.

In programmed current traces, which simulated the channel's inactivation behavior as observed experimentally, we found that the total number of functional channels (n) could be overestimated because assumption iii) is not completely fulfilled. Nevertheless, the values calculated for p and P_S were similar (within 10%) to the programmed values for the correct n. To decide among possible values of n calculated by the maximum likelihood estimator, a test was found, based on the maximum number of channels that open in each sweep. The probability that n_{max} channels open in the ith sweep is at given n_{Ai}

$$p(n_{max}|n_{Ai}) = \left[\sum_{k=0}^{n_{max}}\binom{n_{Ai}}{k}p^k(1-p)^{n_{Ai}-k}\right]^{T_{ges}} - \left[\sum_{k=0}^{n_{max}-1}\binom{n_{Ai}}{k}p^k(1-p)^{n_{Ai}-k}\right]^{T_{ges}} \tag{9}$$

and thus, the probability of n_{max} is

$$p(n_{max}) = \sum_{n_{Ai}=n_{max}}^{n} p(n_{max}|n_{Ai}) \cdot \binom{n}{n_{Ai}} \cdot P_S^{n_{Ai}} \cdot (1-P_S)^{n-n_{Ai}} \tag{10}$$

The theoretical distribution $p(n_{max})$ was first determined for the possible values of n. Then, the empirical distribution of n_{max} was determined, and the χ^2 test (Papoulis, 1991) with $n-2$ degrees of freedom (if $n > 2$) was used to find the theoretical distribution that shows the best agreement with the empirical distribution. The procedure can be reasonably applied for the analysis of multi-channel data with $n < 9$, as established with programmed current traces.

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