Cell Dissociation with Papain Reduces the Density of cGMP-Activated Channels of the Retinal Rod

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Abstract A proteolytic enzyme, papain, is the enzyme commonly used for dissociation of retinal neurons. To ask whether papain modifies the activity of ion channels, cGMP-activated channels were recorded in inside-out membrane patches excised from the bullfrog rod outer segment, and the activity was compared between cells dissociated using papain and cells dissociated mechanically. While the affinity to cGMP, *I-V* relation, channel kinetics, and single-channel current amplitude were almost the same in both papain-treated and the control preparations, the total current flowing through the patch membrane of the papain-treated cells was reduced to about 20% of the control, suggesting that papain reduced the density of cGMP-activated channels.

Key words: papain, cell dissociation, ion channel, rod photoreceptor, retina.

Solitary neurons dissociated from the retina have been used extensively for patch clamp studies and have provided important information on ion channels and transmitter receptors of retinal neurons. The proteolytic enzyme, papain, has been used most commonly for dissociation of retinal neurons because it yields healthy solitary cells both morphologically [1, 2] and physiologically [3–5]. However, whether or not papain destroys or modifies channel or receptor molecules and alters their activity has been debated. It is the aim of the present study to examine the effect of cell dissociation with papain on the activity of ion channels. To compare cells isolated by papain treatment with those isolated without using a proteolytic enzyme, we chose the cGMP-activated channels of the bullfrog rod photoreceptor as the preparation because photoreceptors are the only retinal cell that can be isolated mechanically. We found that papain treatment reduced the number of active channels, but the remaining channels seemed to function normally.

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MATERIALS AND METHODS

Preparation. Experiments were performed on membrane patches obtained from rod outer segments of the bullfrog (*Rana catesbeiana*). All procedures for cell preparation were carried out under the room light. Animals were sacrificed by decapitation and double-pithed. After the eyes were enucleated and hemisected, the retina was detached from the pigment epithelium. Cell dissociation was achieved according to the protocol in Fig. 1. In the following description, the preparation using papain will be referred to as preparation P and the two types of mechanically isolated control preparations will be referred to as preparation M1 and M2. To

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Fig. 1. Procedures of cell dissociation. It took less than 30 min for M1 and 1.5–2 h for M2 and P.

make preparation P, the retinas were cut into 1-mm wide strips and incubated in Ringer solution (with a composition of 111 mM NaCl, 2.5 mM KCl, 1.0 mM CaCl_2 , 1.6 mM MgCl_2 , 10 mM glucose, and 5 mM Tris, pH=7.8) containing 10 U/ml papain (Worthington) and 1 mM cysteine for 20 min at 28°C. The papain concentration in the present work was within the range used to dissociate retinal neurons (3–15 U/ml, cf. [6]). After enzyme treatment, the retinal pieces were rinsed several times in papain-free Ringer solution and were triturated with a large bore fire-polished Pasteur pipette. This procedure usually took 1.5 to 2 h.

To make M1, the retina was minced with a razor blade in the Ringer solution without enzyme [7]. This procedure took less than 30 min. To make preparation M2, we used the identical procedure used to make P but no enzyme was used. Thus, to make M2 it took about 1.5 to 2 h. Some cells of P had an axon terminal but most M1 and M2 cells had no axon terminal. Otherwise, there was no morphological difference among cells of P, M1, and M2. Recording was made either from cells having the inner segment or from detached outer segments, but the results were almost identical. Experiments were performed under room light and at room temperature (20–25°C).

Electrodes and recording system. Patch pipettes were made of Pyrex tubing (outer diameter, 1.5 mm, inner diameter, 0.9 mm). Pipette resistance measured in the Ringer solution was about 20 M Ω (range 15 to 25 M Ω). Pipettes were filled with a solution containing (in mM): NaCl, 117.5; EDTA, 0.15; and Tris, 5.0 (pH=7.8). The concentration of Ca^{2+} and Mg^{2+} was reduced by adding EDTA to eliminate blocking of cGMP-activated channels by divalent cations [7–9]. A Ringer-agar bridge connected the bath to the grounded Ag-AgCl wire. The output of a patch-clamp amplifier (List-Electronic L/M-EPC7) was stored on an FM magnetic tape (Sony Magnescale A-45) with a bandwidth of 0–10 kHz (+0.5 to –1 dB) for off-line analysis.

Recording procedures. Gigaseal (usually 20–50 M Ω) was attained when a gentle suction was applied to the pipette touching the surface of a rod outer segment [10]. There was no apparent difference in the ease of obtaining gigaseal among the three preparations. Suction did not cause any “bleb” formation [11] at the pipette tip. The inside-out patch was obtained by gentle tapping of the manipulator. Solutions identical to the pipette solution but with or without additional cGMP (Sigma) were gravity-fed and perfused the cytoplasmic side of the membrane [12].

Data analysis. To calculate the power spectrum distribution records were filtered with an 8-pole Butterworth filter (NF Electric P-84; cut-off frequency, 4 kHz) and digitized by a 12-bit A/D converter attached to a computer (Compaq XE 466) at 10 kHz. To obtain single channel events, data were sampled at 10 kHz after passing through an 8-pole Bessel filter with a cut-off frequency of 2 kHz. For calculation of the variance, records were filtered with an 8-pole Bessel filter (cut-off frequency; 4 kHz) and sampled at 1 kHz. Records were also reproduced on a thermal array recorder (Graphtec WR7600; frequency range, 0 to 500 Hz) after filtering through a low-pass Bessel filter (cut-off frequency; 200 Hz).

RESULTS

Maximum conductance of the patch

Application of a saturating concentration of cGMP (1 mM) to the cytoplasmic side of the patch membrane evoked an inward current (Fig. 2, membrane potential, -20 mV), similar to previous reports (cf. [13]). The current was accompanied by an increase in current fluctuation. Conductance increase generated by cGMP was calculated from the recorded current (after subtracting the leak current) and the membrane potential (-20 or -40 mV). Figure 3 shows the distribution of the conductances generated by a saturating dose of cGMP, referred to here as the maximum conductance ($\gamma_{\max}$) in preparations M1, M2, and P. $\gamma_{\max}$ in P showed a distribution skewed to the lowest bin (0.6 ± 0.15 nS, mean $\pm$ SEM, $n=42$, Fig. 3C upper histogram), and was different from that in M1 (3.6 ± 0.5 nS, $n=18$, Fig. 3A),

Fig. 2. Sample records of the current evoked by a saturating concentration of cGMP (1 mM). Membrane potentials, -20 mV; bandwidth, 0–200 Hz. (A) M1, (B) M2, (C) P.

Fig. 3. Distributions of the maximum conductance of the patch. (A) M1, (B) M2, (C) P. Upper histograms show full distributions and lower histograms are expanded view of distributions at maximum conductances less than 1 nS (numbers of patches with maximum conductances larger than 1 nS are indicated as the right most columns).

and M2 (2.7 ± 0.8 nS, $n = 16$, Fig. 3B). Samples having an $\gamma_{\max}$ of less than 1 nS comprised 83% in P, 22% in M1, and 31% in M2. The difference is more clearly recognized in the lower histograms in Fig. 3. $\gamma_{\max}$ was less than 0.2 nS in nearly half of the P samples (20 out of 42). $\gamma_{\max}$ in P was significantly smaller than those in M1 or M2, while there was no significant difference between M1 and M2 ($p < 0.01$, Wilcoxon's rank sum test [14]).

It might be argued that the current amplitude depends on the size of the patch membrane that is determined by the diameter of the patch pipette. However, it is unlikely that the observed difference in $\gamma_{\max}$ was caused by a difference in the patch membrane area, since the pipettes were constructed in the same way, and excised patches were formed in the same manner in M1, M2, and P (see MATERIALS AND METHODS). Also, a lower limit on patch area is set by the area of the pipette lumen (about $0.8 \mu\text{m}^2$). If the M1 and M2 patches were larger than P patches by an amount sufficient to account for the difference in maximal cGMP-activated conductance, it would require that a minimum of $3.6 \mu\text{m}^2$ of the membrane enters the patch pipette at the recording site, producing a readily visible "bleb" and constriction of the outer segment. No such "bleb" or constriction was observed in any of the M1, M2, or P patches.

Dose-response relation

The dose-response relation of the cGMP-induced current showed no differences among preparations M1, M2, and P; similar half-saturating concentrations, $K_{1/2}$ (mean $\pm$ SEM; M1, $20.5 \pm 2.7 \mu\text{M}$, $n = 6$; M2, $28.3 \pm 2.9 \mu\text{M}$, $n = 3$; P, $27.5 \pm 2.9 \mu\text{M}$, $n = 4$) and similar Hill coefficients (approximately 2 for all 3 types of preparations). The results are summarized in Fig. 4. To compare across the data, current was normalized by the maximum current, $I_{\max}$. The solid lines through the data in Fig. 4 were drawn according to the Hill equation:

$$I/I_{\max} = G^2 / (1 + G^2), \quad (1)$$

where I represents the current amplitude, G is the cGMP concentration divided by the averaged $K_{1/2}$. As there was no difference in $\gamma_{\max}$ and agonist binding between M1 and M2, further comparison was made between M1 and P.

I-V relation

I - V relations of the response to a saturating dose of cGMP (1 mM) are shown in Fig. 5. Similar to the previous report [15] the I - V relation of M1 in a solution without divalent cation was almost linear (Fig. 5, open squares). The I - V relations obtained from patches of papain-treated cells (P) were also almost linear (Fig. 5, filled squares and filled circles). Thus, it is inferred that papain treatment did not affect the I - V relation.

Power spectrum distribution

Papain treatment reduced $I_{\max}$ but had no effect on either the dose-response or

Fig. 4. Dose-response relations to cGMP in three preparations. Lines were drawn according to the Hill equation of $I/I_{\max} = G^2/(1+G^2)$, where I is the current, $I_{\max}$ is the maximum current evoked by a saturating concentration of cGMP (1 mM), and G is the concentration of cGMP divided by the averaged $K_{1/2}$ (M1, 20.5 μM ; M2, 28.3 μM ; P, 27.5 μM).

Fig. 5. I - V relations of the response to a saturating dose of 1 mM cGMP (open squares: M1, filled squares and filled circles: P).

I - V relations, as described above. Therefore, the reduction of $I_{\max}$ must be caused by either 1) changed kinetics of the channel, 2) a decreased single-channel current, 3) reduced number of active channels in the patch, or 4) combination of these factors. Channel kinetics were estimated by power spectrum analysis. Figure 6 shows the power spectrum distribution of cGMP-induced current in M1 and P. The power spectrum distribution was obtained by subtracting the spectrum distribution in the absence of cGMP from that in the presence of 1 mM cGMP. As shown in Fig. 6, the power spectrum density of both M1 and P can be described by the sum of 2 Lorentzian functions (corner frequencies: M1, 60 Hz and 1.9 kHz; P, 63 Hz and 2.2 kHz) in agreement with previous reports [8, 16]. Nearly identical values of corner frequencies for both preparations are an indication that papain treatment did not change the channel kinetics. However, the power spectrum of P is smaller than that of M1 by about one order. This difference between the power spectra, therefore, suggests that single channel current was smaller in P, or that the channel density was lower in P.

Single channel current

To observe single channel currents, records were obtained under perfusion of a low concentration of cGMP ($\leq 10 \mu\text{M}$). The channels in both M1 and P showed similar activity (Fig. 7A₁, B₁). However, both in M1 and P flickering similar to previous reports was observed even in lowered divalent cations [8, 9, 17], and it was difficult to identify single-channel events in the raw data. As an alternative method, amplitude histograms were plotted (Fig. 7A₂, B₂). The distribution deviated from

Fig. 6. Power spectrum density of noise evoked by 1 mM cGMP for M1 (open squares) and P (filled squares). Spectrum of background noise was subtracted in each case. Power spectra were calculated from 102.4-ms samples of membrane current and were averaged 45–58 times (membrane potential was -20 mV), digitized at 10 kHz after low-pass filtering at 4 kHz (8-pole Butterworth type). Data points are averages of individual frequency points as follows: < 200 Hz, 2 points; 200–500 Hz, 5 points; > 500 Hz, 10 points. Lines show sum of 2 single Lorentzian functions. Corner frequencies: M1, 60 Hz and 1.9 kHz; P, 63 Hz and 2.2 kHz.

the Gaussian curve that represents the baseline fluctuations. The difference between the obtained data and the Gaussian curve, which is likely to represent the distribution of current amplitudes in the open state, was replotted in Fig. 7A₃ (M1) and B₃ (P). The differences can also be fitted by another Gaussian curve with a peak amplitude of -0.87 and -0.82 pA, respectively (membrane potentials: M1, -96 mV; P, -81 mV). As the cGMP-activated channel shows rapid flickering, the peak amplitude obtained from the amplitude histogram could be underestimated in both cases [18]. However, as described in the previous section kinetics of the single channel of both M1 and P showed similar properties. Thus, the data suggest that papain treatment did not alter the amplitude of the single-channel current.

Fig. 7. A_1 and B_1 : Single channel activities obtained by perfusion of a low concentration of cGMP (A_1 : M1, 2 μ M; B_1 : P, 10 μ M). Dashed lines indicate the baseline level without cGMP. Bandwidth, 0–2 kHz. Membrane potential: M1, –95 mV; P, –80 mV. A_2 and B_2 : Amplitude histograms obtained under perfusion with 2 μ M (M1, A_2) or 10 μ M (P, B_2) of cGMP. Membrane potential: M1, –96 mV; P, –81 mV. Bandwidth, 0–2 kHz. Dashed lines show Gaussian curves with standard deviations of 0.42 pA (M1) or 0.38 pA (P). A_3 and B_3 : Differences between points and Gaussian curves in A_2 (M1) and B_2 (P). Dashed lines are Gaussian curves centered at –0.87 pA (M1) or –0.82 pA (P) and have a standard deviation of 0.43 pA (M1) or 0.42 pA (P).

Density of the channels

The density of channels in a patch was estimated from the relation between the mean current amplitude, I_{mean} , and the variance of cGMP-induced current. Measurements were made during washout of 1 mM cGMP. Figure 8A shows sample recordings. As shown in the upper traces in Fig. 8A which are a.c.-amplified records of the lower traces, the noise level peaked during washout of 1 mM cGMP. Although I_{max} of P was smaller than that of M1, data from M1 (Fig. 8B, C, open squares) and P (filled squares) were well-fitted by a parabola from the relation between the variance σ^2 and I_{mean} :

$$\sigma^2 = iI_{\text{mean}} - (I_{\text{mean}})^2/N, \quad (2)$$

where i is the single-channel current amplitude and N is the number of channels in the patch. Even at the saturating concentration of cGMP, σ^2 did not become zero. To obtain a best fit to the data of Fig. 8 (membrane potential, -20 mV), we selected the following values: i , 0.38 pA for M1 and 0.31 pA for P; N , 190 for M1 and 38 for P. These results suggest that the density of active channels was decreased in the papain-treated preparations while the single-channel current amplitude remained unchanged (see also Fig. 7).

DISCUSSION

We report here that the density of active channels in the patch was decreased significantly by papain treatment, while the properties of remaining active individual channels were unaffected. Although the total current flowing through the patch membrane was reduced to about 20% of the control, the affinity to cGMP, I - V relation, the channel kinetics, and single channel current amplitude were almost the same in both papain-treated and control preparations.

The present study demonstrated that papain reduces the number of channels, perhaps destroying or modifying the channel molecule. Surprisingly, however, there is also a report that papain treatment uncovered or activated a new channel protein. Hestrin and Korenbrot [19] found that voltage-activated potassium channels appeared in the plasma membrane in the papain-treated rod outer segment, while voltage-activated channels were not seen in the outer segment membrane of the mechanically dissociated preparation. Although molecular mechanisms of channel protein modulation are unknown, it is reasonable to assume that, during cell dissociation, papain acted from the extracellular surface. Since the affinity of the channel to cGMP was not affected, it seems possible that papain did not affect the cGMP binding site. Perhaps, some extracellular site important for ion passage could be affected. However, it is also possible that some change at site(s) on the extracellular surface modified the configuration of the channel, inhibiting ion passage and/or cGMP binding.

Papain has been frequently used to dissociate retinal neurons which have been used in the study of voltage-activated and ligand-gated channels. These studies

provided new knowledge on ion channels and receptor molecules of retinal neurons and the fruitful results indicate that papain treatment does not damage all types of channels or receptors. However, the present study shows that at least the cGMP-activated channel of the rod photoreceptor suffers significant damage, though not total destruction. Cyclic GMP-activated channels are also reported to exist in ON-type bipolar cells [20, 21] and it is possible that dissociation of such cells by papain treatment reduces the density of cGMP-activated channels. Thus, dissociation conditions such as the enzyme concentration and the incubation time should be carefully specified, and the results obtained in such studies should be evaluated in reference to data obtained by other methods such as slice preparation. However, isolated cell preparation is still an excellent preparation.

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Fig. 8. A: Sample records showing change in the current noise during washout of 1 mM cGMP. Upper traces show a.c.-amplified records of the lower records. B: Relation between the mean current amplitude and the variance. Data were obtained from M1 (open squares) and P (filled squares) patch. Variance obtained from the current signal in the absence of cGMP was subtracted. The continuous lines indicate a parabola described by the formula, $\sigma^2 = iI_{\text{mean}} - (I_{\text{mean}})^2/N$, where σ^2 is the variance, i is the single-channel current amplitude, and N is the number of channels in the patch. C: Relation between σ^2/I_{mean} and I_{mean} . The straight lines were drawn according to the formula, $\sigma^2/I_{\text{mean}} = i - I_{\text{mean}}/N$. The intercept with the ordinate corresponds to i with a slope $-1/N$. Membrane potentials, -20 mV. i , 0.38 pA for M1 and 0.31 pA for P; N , 190 for M1 and 38 for P.

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