Inactivation in *Shaker*B K^+ Channels: A Test for the Number of Inactivating Particles on Each Channel

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ABSTRACT Fast inactivation in *Shaker*B K channels results from pore-block caused by "ball peptides" attached to the inner part of each K channel. We have examined the question of how many functional inactivating balls are on each channel and how this number affects inactivation and recovery from inactivation. To that purpose we expressed *Shaker*B in the insect cell line Sf9 and gradually removed inactivation by perfusing the cell interior with the hydrolytic enzyme papain under whole cell patch clamp. Inactivation slows down as the balls are removed by an amount consistent with the presence of four balls on each channel. Recovery from inactivation has the same time course early and late in papain action; it does not depend on the number of balls remaining on the channel, consistent with the idea that reinactivation is not significant during recovery from inactivation. Our conclusion is that *Shaker*B has four ball peptides, each capable of causing inactivation. Statistically, the balls are identical and independent. The stability of N-type inactivation by the remaining balls is not appreciably affected by removing some of the balls from a channel.

INTRODUCTION

Voltage-dependent potassium and sodium channels are thought to be formed by the insertion in the membrane of four subunits or domains, arrayed with fourfold symmetry around a central pore (Noda et al., 1986). In the case of Na channels this topological arrangement originates from the four internal repeats of a single polypeptide (Noda et al., 1986). K channels instead are formed by four independent polypeptides or subunits, each about one-fourth the size of the main subunit of Na channels (Jan and Jan, 1990; MacKinnon, 1991).

Among voltage-dependent K channels, *Shaker*B gives rise to currents with the fastest inactivation (Iverson and Rudy, 1990). It is thought that *Shaker*B has two types of inactivation: a fast, or N-type, and a slow, or C-type, inactivation. Slow inactivation develops with a time constant of seconds after a depolarization and is related to an extracellular domain of the protein (Grissmer and Cahalan, 1989; Choi et al., 1991; Hoshi et al., 1991). Fast inactivation, by contrast, develops with a time constant of milliseconds and has been shown to be produced by the block of the pore by a "ball" formed by the NH_2 terminus of the protein (Hoshi et al., 1990, Ruppersberg et al., 1991; Demo and Yellen, 1991).

In the ball-and-chain model of inactivation proposed for Na channels, a ball diffuses into the inner mouth of an open channel to inactivate it (Armstrong and Bezanilla, 1977; Armstrong, 1981). Removal of inactivation with the proteolytic enzyme pronase showed that the rate of inactivation does not change as inactivation is removed (Armstrong et al., 1973). This is consistent with the presence of a single inactivation particle attached to each channel. When the ball is removed, the channel does not inactivate, but channels not yet affected by the enzyme inactivate at the normal rate. Based on structure, one would expect K channels to have four equivalent balls (see above), although only one is needed to inactivate the channel. If so, *Shaker*B inactivation, unlike the inactivation of squid Na channels, should slow down as the average number of balls per channel decreases due to enzyme action.

We have used the approach of gradually removing inactivation with intracellular papain to estimate the number of functional inactivation particles on K channels and to examine how this number affects inactivation and recovery from inactivation. Our conclusions are: (1), fast inactivating K channels have four balls or inactivating particles; (2), the balls seem to be equivalent and independent in their statistical behavior; (3), the stability of the N-type inactivation does not depend on the number of inactivating balls; and (4), reinactivation is not significant during recovery from inactivation.

MATERIALS AND METHODS

Cell culture and *Shaker*B expression

Sf9 cells were kept in culture at 27°C, in Grace's medium supplemented with 10% fetal bovine serum and with a combination of penicillin (50 units/ml), streptomycin (50 μg/ml), and gentamycin sulfate (50 μg/ml). For the patch clamp experiments, the cells were plated onto coverslips and infected with the recombinant baculovirus *Autographa californica* nuclear polyhedrosis virus, containing the *Shaker* H4 K^+ channel cDNA (Klaiber et al., 1990). The cells were kept at 27°C and used for experiments 2 days after the infection. The recombinant baculovirus was a kind gift of Dr. C. Miller.

Electrodes and recording

The electrodes were pulled from borosilicate glass (KIMAX 51), fire polished to a 0.7–1.3 MΩ resistance, and used without any further treatment.

Macroscopic currents were recorded under whole cell patch clamp (Hamill et al., 1981) 2 days after the infection of the cells. *Shaker*B channels

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provided the only K^+ conductance present in the plasma membrane of the infected Sf9 cells (Klaiber et al., 1990).

Approximately 80 to 100% of the series resistance was electronically compensated. The holding potential used throughout the work was −80 mV. Leak current was measured with control pulses of −50 mV from the holding potential of −80 mV.

Solutions

The composition of the internal solution used in all the experiments was 90 mM KF, 30 mM KCl, 2 mM $MgCl_2$, 10 mM EGTA, and 10 mM HEPES-K, pH 7.2. Papain (Sigma Chemical Co, St. Louis, MO) was dissolved in this solution at 0.3 mg/ml (14 units/mg). The normal external solution was 145 mM NaCl, 2 mM $CaCl_2$, and 10 mM Mes-Na, pH 6.4. In some cases, KCl and/or $CaCl_2$ were substituted for NaCl to achieve a higher $[K^+]$ or $[Ca^{2+}]$, as indicated.

RESULTS

Removal of inactivation by papain

The addition of the proteolytic enzyme papain to the medium in the patch pipette selectively removes the fast, or N-type, inactivation (I_n) of *Shaker*B in the whole cell mode (Hoshi et al. (1990) used trypsin for the same purpose). In our conditions, removal of inactivation began ~3 minutes after breaking into the cell and was complete in 10 to 15 minutes. Fig. 1 illustrates the general features that accompany the removal of inactivation by comparing the currents through the channels at three stages of modification in a representative cell. Similar changes have been observed in all the cells that we have treated with intracellular papain (more than 20). At each voltage, the bottom traces show current through unmodified channels, taken just after the establishment of the whole cell configuration; the middle traces show the currents at an intermediate stage of modification; and the uppermost traces were taken when most of the inactivation has been removed (>90%). Destruction of fast inactivation is marked by an increase in current at the end of the depolarizing pulse and by a corresponding increment in the amplitude of the inward tail currents at the moment of the repolarization.

The traces in Fig. 1 show that inactivation removal also causes an increase in the amplitude of the outward current at each voltage. The increment in the amplitude of the peak current is voltage dependent. That is, maximum current during the pulse increases 10-fold at −20 mV but only 1.3-fold at +60 mV. This effect has not previously been reported for K channels but has been documented in Na channels (see Discussion). The increase in the amplitude of the current is a consequence of the destruction of fast inactivation: more channels populate the open state because the inactivated state is less available.

Inactivation slows down as it is being removed

At +70 mV, the unmodified channels inactivate with the time course of a single exponential, with a time constant (τ) of 1.6 ± 0.1 ms (n = 11). The first visible change during perfusion with papain is the slowing of inactivation (observed in more than 20 cells). The slowing begins ~3 minutes after breaking into the cell, as shown in Fig. 2*A*, which compares currents at +70 mV at various stages of papain action. The trace with the fastest inactivation corresponds to the unmodified channels (τ = 1.6 ms in this case), taken shortly after breaking into the cell. The decay of the current becomes progressively slower as papain acts.

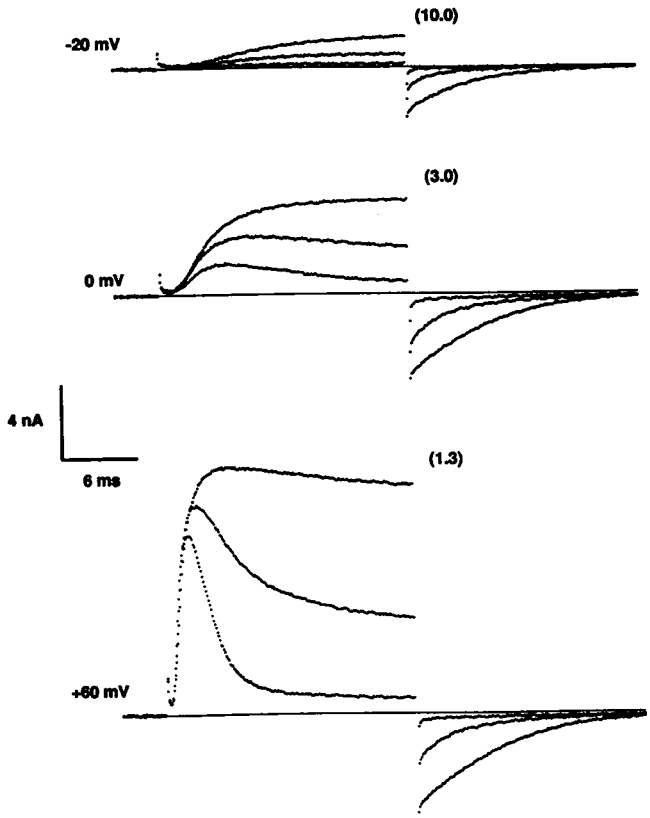


FIGURE 1 Removal of inactivation by papain. K^+ currents were elicited by 20 ms depolarizations to the indicated values, from a holding potential of −80 mV. In each panel, the lowermost trace was taken just after the establishment of the whole cell configuration and shows current through unmodified channels. The middle trace shows the currents when about half of the inactivation had been removed. The inward tail currents are larger, as is the outward current. The top trace at each voltage was taken after most of the inactivation had been removed. The numbers in parentheses are the increase in the maximum current amplitude, relative to the unmodified channels. Papain concentration in the internal solution was ~0.3 mg/ml. External solution: 30 K^+ + 1 Ca^{2+}.

The slowing of inactivation excludes the possibility that *Shaker*B has only one inactivating ball. If there were only one ball, the current at any stage would be given by the contribution of only two populations: the channels that still have a ball attached and inactivate at their normal rate plus the channels that no longer have a ball and therefore do not inactivate. Thus, as the removal of inactivation progresses there would be no change in the time constant of inactivation, only a decrease in the fraction of the channels that inactivate. The pattern of inactivation shown in Fig. 2 shows clearly that each *Shaker*B channel must have more than one inactivating ball. Significantly, slowing begins while inactivation is still virtually complete, which shows that not all of the balls must be present for the channel to inactivate.

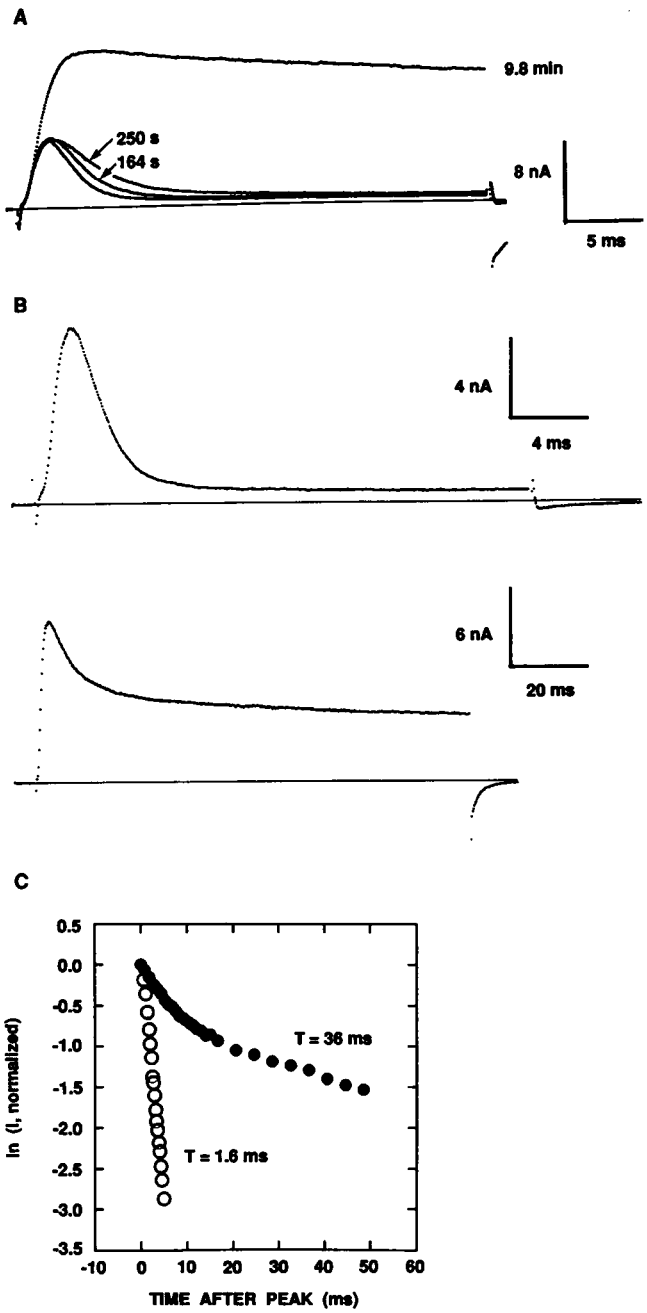


FIGURE 2 Inactivation slows as papain acts. (*A*) I_K was elicited by a 70 mV/30 ms pulse from a holding potential of −80 mV at various stages of papain action. The current with the fastest inactivation corresponds to the unmodified channels, with τ = 1.6 ms. The numbers show the time in the whole cell after the recording of the control trace. Notice that the inactivation slows substantially before there is any increase of the steady current. (B, C). The logarithm of the decaying phase of I_K, after subtracting the steady current, is plotted against time for the two traces in *B*. The open circles are from the trace taken before papain action and the filled circles from the trace after papain had removed more than half of the inactivation. Current decay is a single exponential initially and becomes multiexponential as papain acts, including a slow phase with τ of 36 ms.

The time course of inactivation is plotted on a semilogarithmic scale in Fig. 2*C* for the two traces in Fig. 2*B*. Before papain has acted, inactivation is a single exponential with a time constant of 1.6 ms. After more than half of the inactivation has been removed, the initial phase is slowed, and there is a final phase that is very slow, with a time constant of 36 ms.

*Shaker*B channels have four inactivating balls

Fig. 3 compares the observed inactivation of the current with the expected pattern for channels with one to four balls. The simulation was done under the simplest hypotheses: that the balls are equivalent and independent, that they are removed independently, and that a single ball is sufficient to cause inactivation. In practice, the activation gating of the channel was determined, first, by fitting a trace acquired after papain had destroyed most or all of fast inactivation. The kinetic scheme used was the following:

$$C_5 \xrightarrow{3\ ms^{-1}} C_4 \xrightarrow{3\ ms^{-1}} C_3 \xrightarrow{3\ ms^{-1}} C_2 \xrightarrow{3\ ms^{-1}} C_1 \xrightarrow{2\ ms^{-1}} Open \xrightarrow{k_i} Inactivated$$

The experimental trace and the fit are shown in the upper traces of Fig. 3*A*. There remains a slow inactivation, as shown in Fig. 2*B*. Although we do not know the origin of this slow decay of the current, we tentatively ascribe it to inactivation caused by cut off balls that are floating free in the cytoplasm. It is much too fast to be accounted for by C-type inactivation.

After determining the rate constants for activation (the values shown were one of many combinations that gave an adequate fit) and the scale factor for current amplitude, these parameters were frozen for all the other simulations. We first fit inactivation before papain action (Fig. 3*A*, lower traces). For this trace, k_i was 0.6 ms^{-1}, and the rate of inactivation due to cut off balls was zero, as none had been cut off. The peak current is not perfectly fit, but the decay of the current is well reproduced.

After papain action begins, the distribution of channels having, e.g., four, three, two, or one ball remaining can be

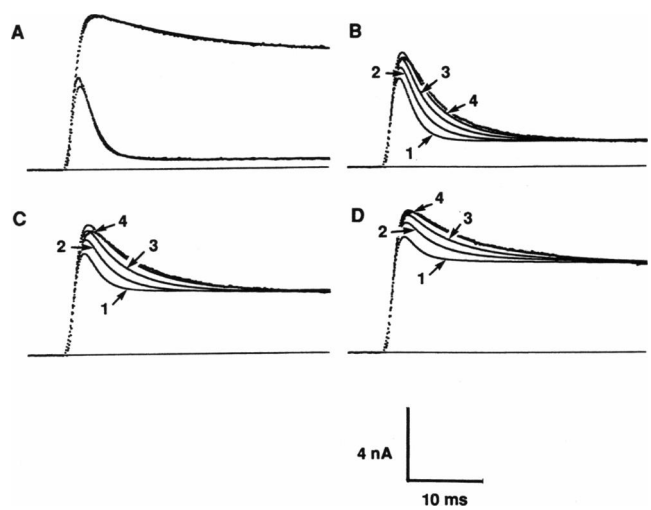


FIGURE 3 The experimentally observed time course of inactivation as papain acts is compared with the expected time course if there are four, three, two, or one inactivating ball per channel, as described in the text.

estimated from the steady-state current by using the binomial distribution (see Discussion). If, for example, there are originally four balls and 15% of the channels no longer inactivate (Fig. 3*B*), the distribution is 2% with four balls, 13% with three, 33% with two, 36% with one, and 15% with none. Each of these separate groups was represented by a kinetic scheme of the type shown above, with k_i appropriate to the number of balls remaining (see Discussion). For example, channels with four balls inactivated at the rate of 0.6 ms^{-1}, channels with three at the rate of 0.75 × 0.6 ms^{-1}, etc. To this inactivation rate was added the rate due to the balls free in the cytoplasm, which was proportional to the calculated number of balls deleted. The contribution of each group to the total current was equal to the fraction of channels with that number of balls (see Discussion).

Fig. 3, *B–D*, shows the experimental results as papain acts, together with the theoretical traces calculated by assuming four, three, two, and one inactivating ball originally present on each channel. The fit for one ball is very poor and, for the reasons given above, fails to reproduce any slowing of the inactivation as it becomes less complete. The fit improves as the number of balls originally present on a channel increases to two, three, and four. The fit is clearly the best for four, leading us to conclude that each channel initially has four inactivation balls.

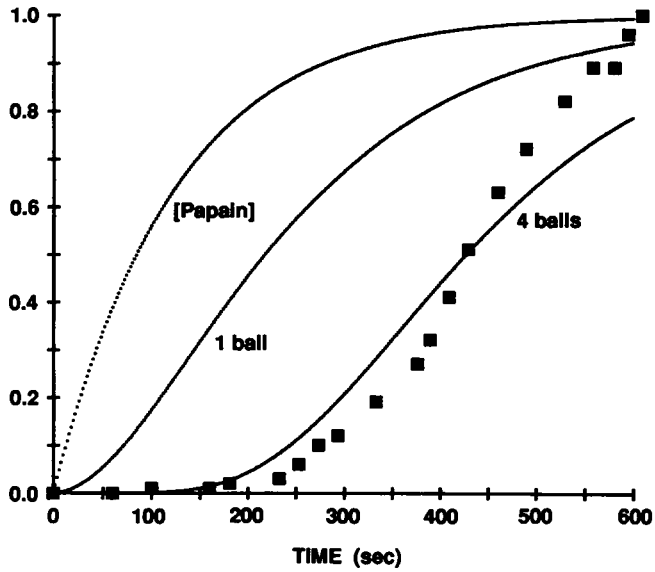


FIGURE 4 Increase of the steady current as papain acts. The filled squares show the time course of the normalized current at the end of a long pulse as a function of time after breaking into the cell. The [papain] curve is theoretical and calculated from the access resistance as described by Pusch and Neher (1988). The curves labeled *1 ball* and *4 balls* are theoretical curves, calculated on the assumption that 1), papain acts at a rate proportional to its concentration, and 2), that only one ball must be removed to destroy inactivation or that all of four identical balls must be removed.

The time course of papain action

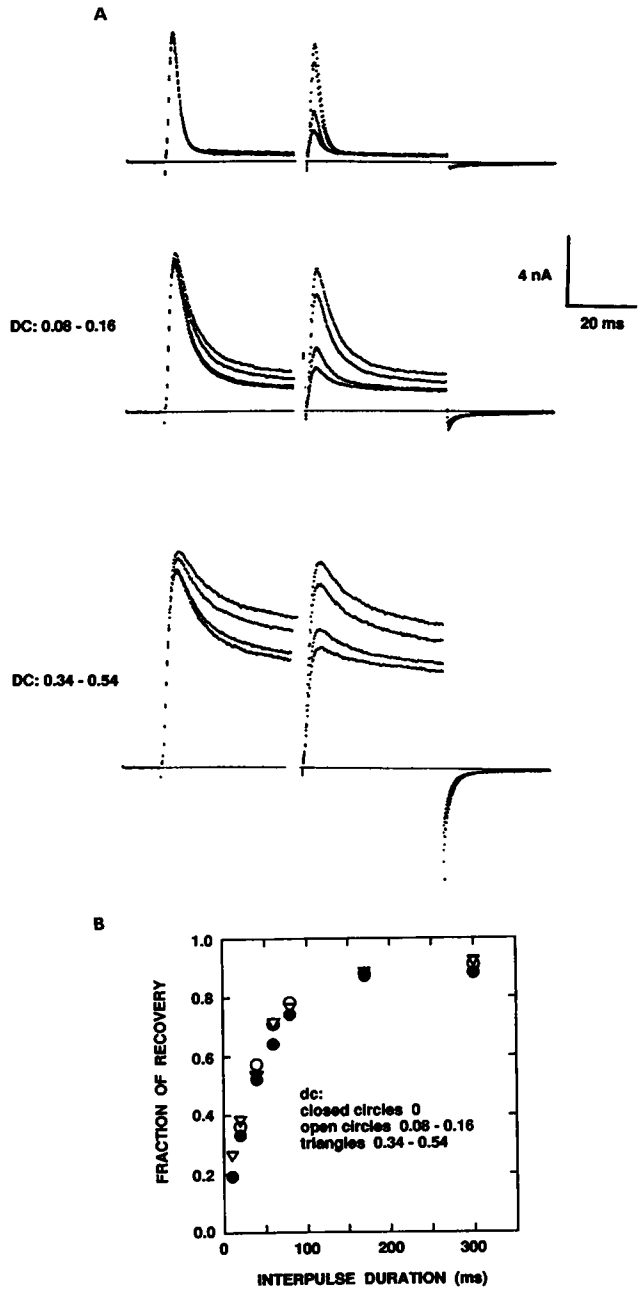
Somewhat more qualitative support for the conclusion that *Shaker*B channels have four balls, or at least several, comes from an examination of the time course of the increase of the noninactivating current in papain. We assume that the rate of removal of balls is proportional to the papain concentration in the cytoplasm. Expectations regarding the time course of inactivation removal are most easily explained for an idealized case in which the papain concentration instantly rises to its final value in the cytoplasm. The time course of the increase of the noninactivating current then depends on the number of balls present on each unmodified channel. For a single ball, the time course would be exponential, whereas if there are several balls per channel, the time course would be S-shaped, inasmuch as a channel continues to inactivate, albeit more slowly, until the last ball is removed.

More realistically, if the papain concentration rises with a slow but predictable time course in the cytoplasm, the time course of inactivation removal can be estimated computationally. Papain diffusion into the cell was calculated with Eqs. 9 and 18 of Pusch and Neher (1988) for a cell with an access resistance of 1.4 MΩ and capacitance of 18 pF. Fig. 4 shows the estimated time course of the papain concentration in the cytoplasm, and the filled squares give the steady current (measured at the end of a long pulse) as a function of time. The curve labeled *4 balls* was calculated on the assumption that all four balls must be removed for a channel to carry steady current, with an empirically determined rate constant for papain removal of the balls (0.00001 s^{-1} $(mg/ml)^{-1}$). The fit is not excellent, but the same calculation for one ball gave a much poorer fit, and it is clear that, for one ball, no value of the rate constant for papain action would give a fit as good as four balls. (The *1 ball* curve is sigmoid because of the slow rise of papain concentration in the cell.) Were there more certainty regarding the papain concentration, a better fit could probably be obtained.

Recovery from inactivation in modified channels

During recovery from fast inactivation, the channels reflux from the ball-occluded state to the open state and then to the closed states (Zagotta and Aldrich, 1990; Demo and Yellen, 1991; Ruppersberg et al., 1991). Changing the number of balls per channel enzymatically could theoretically alter recovery in at least two ways. First, if the inactivation rate is high, the channels might reinactivate during recovery and would be less likely to do so if there were fewer balls per channel. Second, the ball occluding the channel might be more, or less, stably bound if more balls are present per channel.

We tested these possibilities by measuring recovery from inactivation with a standard two-pulse protocol, at three stages of the removal of inactivation by papain (Fig. 5). To speed recovery, the cell was in 40 mM K. (Determination of the recovery time course was a slow procedure, and in the two later determinations an increase in the steady current is apparent as each series proceeds.) The plots in Fig. 5*B* show that the recovery curves almost superimpose for the three determinations despite the changing distribution of balls per



channel, estimated by the pie charts in Fig. 5*C* (see Discussion). This can only mean that the recovery rate is not significantly affected by the average number of inactivating balls on a channel. The fact that the noninactivating current varied in the later determinations does not alter this conclusion.

DISCUSSION

The addition of papain to the patch pipette destroys the fast, or ball-and-chain, inactivation of *Shaker*B in the whole cell configuration (Hoshi et al., 1990). Parallel to the removal of inactivation, the maximal amplitude of the outward current increases as well as the time to peak (Figs. 1 and 2). Both changes can be explained as a direct consequence of the destruction of inactivation, as has been extensively discussed for Na channels (Gonoi and Hille, 1987; Cota and Armstrong, 1989) and for the A-type current of GH_3 cells (Matteson and Carmeliet, 1988; Oxford and Wagoner, 1989). The fractional increase is somewhat variable (Figs. 1 and 2) for unknown reasons.

*Shaker*B has four inactivating balls

We have shown that while papain is acting, the remaining N-type inactivation becomes slower and multiexponential (Figs. 2 and 3). These changes immediately exclude the possibility that inactivation in *Shaker*B channels is like that in squid Na channels, which have only one inactivating particle (Armstrong et al., 1973). An estimate of n, the number of inactivation particles on an unmodified channel, was obtained in Fig. 3, in which we simulated inactivation during papain action for four values of n. It was assumed that if the channels had n equivalent and independent balls, any one of which was competent to cause inactivation, then the rate k_i for the inactivation step for a channel:

$$O \xrightarrow{k_i} I_n$$

would, after papain, depend on i, the number of balls remaining. The decay of the current would be multiexponential, generated by $n + 1$ populations of channels, and the rate

FIGURE 5 The rate of recovery from inactivation does not change during papain action. (*A*) Currents obtained with a two-pulse protocol at three stages in the removal of inactivation. A 60 mV/40 ms pulse was applied to open and inactivate the channels. The membrane was then repolarized to −80 mV for a variable interval. This interpulse interval, represented by a gap in the trace, was not sampled. A second 60 mV/40 ms pulse was then applied to see the recovery between pulses. The intervals illustrated were 10, 30, 80, and 300 ms. For the two lower sets of traces, papain action is so rapid that the steady current increases from trace to trace. The *DC* at the beginning and at the end of the series is given below the traces. (*B*) The time course of recovery at various stages of papain action from the traces in *A*. The fractional recovery was measured as $(I_{peak,2} - I_{40,2})/(I_{peak,1} - I_{40,1})$, where $I_{peak,2 \text{ or } 1}$ is the peak current in the second and the first pulses, respectively, and I_{40} is the current at the end of the 40 ms pulse. (*C*) The pie charts show the predicted fraction of channels with 0, 1, 2, 3, or 4 balls remaining for the given values of *DC* (0.08, 0.16, 0.34, 0.54), based on the binomial distribution calculations in the text. External solution: 40 K^+ + 40 Ca^{2+}.

constants would have the values:

$$k_n = \left(\frac{n}{n}\right)k_n, k_{n-1} = \left(\frac{[n-1]}{n}\right)k_n,$$
$$k_{n-2} = \left(\frac{[n-2]}{n}\right)k_n, \cdots, k_{n-n} = \left(\frac{[n-n]}{n}\right)k_{in} \quad (1)$$

where k_n is the rate with all of the balls intact. (k_0, or k_{n-n}, has a value of zero, and these channels generate the noninactivating current.) The fraction of the channels with i balls still attached, A_i, was calculated from the binomial distribution:

$$A_i = (n!/[(n-i)!\ i!])p^i(1-p)^{n-i} \quad (2)$$

where p is the fraction of the total balls that are still attached. p can be calculated by the relation derived from Eq. 2:

$$A_0 = DC = (1-p)^n \quad (3)$$

where DC is the fraction of the channels that no longer inactivate, and n, as previously, is the number of balls originally present on each channel. (Eq. 3 is approximate because it assumes that a channel with only one remaining ball will be inactivated 100% of the time in the steady state. According to our data, this is a reasonable approximation.) The total current then was calculated as the sum of $n + 1$ populations of channels, each with its own value of k_i, by using kinetic scheme I given in Results. The relative size of each population was given by A_i. This simple model gives a good fit with $n = 4$, and a distinctly less good fit with $n < 4$.

We got less precise evidence for several balls per channel in Fig. 4, in which we examined the time course of the development of DC current. We hypothesized that the rate of removal of balls is proportional to the concentration of papain multiplied by p, the fraction of balls that remain attached:

$$dp/dt = -\alpha[\text{papain}]p \quad (4)$$

where α is the rate constant of hydrolysis (s^{-1}/mg/ml). To estimate papain concentration in the cytoplasm, we followed Pusch and Neher (1988) and assumed that diffusion was limited by the access resistance. Once in the cytoplasm, equilibration of papain in theory is fast enough to be considered instantaneous. The fraction of channels with all balls deleted, DC, then is:

$$DC = (1-p)^n \quad (5)$$

The comparison of the predictions of Eq. 4 with the experimental data in Fig. 4 shows clearly that one ball cannot fit the data, whereas that with four balls gives a better fit. We did not show the corresponding simulation with an n of two or three balls, because they clearly would have given a less good fit than four.

Two points about the model require comment. The first is the assumption that the balls on a channel are attacked independently, as opposed to the possibility that an enzyme in proximity to the channel would be likely to attack more than one ball while it was nearby. The data clearly show that the attack is independent, requiring four separate hits to remove all of the inactivation balls. This is compatible with the presence in the cytoplasm of appropriate substrate in high concentration. Thus it can be presumed that, after attacking a ball peptide, an enzyme molecule is most likely to be adsorbed by substrate in the cytoplasm. The second point deals with the effect on the calculations of the suggestion that the slow inactivation (τ = 36 ms) arises from cut off balls and is not present initially. The rate of this inactivation is small enough that it has no effect on the conclusions regarding the number of balls.

In summary, Fig. 3 suggests that any of four balls present on an intact channel is sufficient to inactivate the channel. Fig. 4 is compatible with this conclusion. This result is consistent with the findings of Zagotta et al. (1990) that the application of the synthetic ball peptide attached to the cytoplasmic side of the noninactivating ShBΔ6-46, produces a bimolecular block.

The slow component of inactivation cannot originate from channels with balls still attached

At a late stage of papain action, inactivation is quite slow, and the 36-ms time constant cannot be explained by channels with one remaining inactivation ball, unless there are 36/1.6 ~ 23 balls per channel initially, which clearly seems unreasonable. Nor can it be attributed to C-type inactivation, which has a time constant of 1.2 s. One thought is that this inactivation is due to free-floating balls, excised from K channels both in the membrane and from the large reservoir of channels known to be in the cytoplasm. With further papain action, this component gradually disappears, possibly because the free balls are gradually degraded by the continued action of papain.

Recovery from inactivation

Recovery from inactivation is not significantly affected by the average number of inactivating balls in the channels (Fig. 5). This suggests that recovering channels are unlikely to reinactivate before closing. Further, the presence of several adjacent balls on a channel does not seem to stabilize the ball that is involved in inactivating the channel. The finding seems somewhat paradoxical, as the closing rate of the channels is probably less than the inactivation rate, leading to the expectation that some reinactivation would occur during recovery. This point is being examined further.

Since the original submission of this article, an elegant article has appeared that makes the same points regarding inactivation stoichiometry with mutant *Shaker* channels (MacKinnon et al., 1993).

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