Intramolecular and Intermolecular Enzymatic Modulation of Ion Channels in Excised Membrane Patches

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ABSTRACT A calcium-activated potassium channel in posterior pituitary nerve terminals was modulated by phosphorylation and dephosphorylation. Nearly every patch of membrane containing this channel also contained both membrane bound protein phosphatase and membrane-bound protein kinase. By examining the statistical and kinetic nature of phosphorylation and dephosphorylation in excised patches, it was possible to evaluate two contrasting models for these enzymatic reactions. One of these models treated catalysis as an intermolecular process in which the enzyme and substrate are separate molecular species that diffuse and encounter one another during collisions. The second model treated catalysis as an intramolecular process in which the enzyme and substrate reside within a stable macromolecular complex. The study began with a Poisson analysis of the distribution of channel number in patches, and of the number of protein phosphatase-free and protein kinase-free patches. Subsequent kinetic analysis of dephosphorylation yielded an estimate of the mean number of protein phosphatase molecules per patch that was similar to the value obtained from Poisson analysis. Because these two estimates were independent predictions based on the intermolecular model, their agreement supported this model. Analysis of channel number in protein phosphatase-free patches and of the rarity of patches showing partial but incomplete rundown provided additional support for the intermolecular model over the intramolecular model. Furthermore, dephosphorylation exhibited monotonic kinetics with a rate well below the diffusion limit. Thus, several different lines of analysis support the intermolecular model for dephosphorylation, in which the protein phosphatase must encounter its substrate to effect catalysis. In contrast to the monotonic kinetics of dephosphorylation, the phosphorylation reaction exhibited sigmoidal kinetics, with a rate that depended on membrane potential. Voltage dependence is an unlikely property for a kinetic step involving encounters resulting from diffusion. Furthermore, the velocity of the phosphorylation reaction exceeded the diffusion limit, and this observation is inconsistent with the intermolecular model. Thus, both intermolecular and intramolecular enzymatic mechanisms operate in the modulation of the calcium-activated potassium channel of the posterior pituitary. These studies provide a functional characterization of the interactions between enzyme and substrate in intact patches of cell membrane.

INTRODUCTION

Enzymes modulate the activity of ion channels to alter their physiological function, and this modulation contributes to the functional plasticity of the nervous system (Kandel and Schwartz, 1982; Levitan, 1988; Grinnell et al., 1988). Over the past decade, many investigators have focused on enzymatic phosphorylation and dephosphorylation of channel proteins (De Peyer et al., 1983; Hescheler et al., 1987; Lechleiter et al., 1988; Kume et al., 1989; Carl et al., 1991). Recently, several groups have demonstrated that these reactions persist in excised patches and reconstituted lipid bilayers (Chung et al, 1991; Reinhart et al., 1991; Kim, 1991; Kume et al., 1991; Bielefeldt and Jackson, 1994). Thus, some of the enzymes that modulate channels are membrane proteins themselves. In demonstrating a membrane location for enzymes that modulate channels, these observations raise the interesting question of how these proteins interact with one another within the plasma membrane. An enzyme and substrate could associate as two parts of a stable macromolecular complex. It is even possible that the channel proteins themselves could contain the enzymatically active domains, as implied by recent sequence data (Atkinson et al., 1991; Thomas et al., 1991; Jan and Jan, 1992). Alternatively, modulating enzymes could remain apart from their substrates, diffusing within the lipid bilayer, so that catalysis would depend on collisions between the two proteins.

Nerve terminals of the posterior pituitary contain a high conductance calcium-activated potassium channel (BK channel) that is uniquely suited for the regulation of neurosecretion (Bielefeldt and Jackson, 1993) and is strongly affected by phosphorylation (Bielefeldt and Jackson, 1994). We have recently shown that a protein kinase enhances activity of the BK channel, shifting its voltage dependence of activation by about 80 mV to more negative potentials. A protein phosphatase with the pharmacological characteristics of protein phosphatase type 1 reversed this reaction; dephosphorylation reduced channel activity. Both enzymes remained active in excised patches, indicating that they are membrane-bound (Bielefeldt and Jackson, 1994). In the present study, we have exploited this bidirectional regulation of channel activity to investigate how each of these two enzymes interact with the BK channel. We considered two alternative hypotheses, the first of which takes catalysis as an intermolecular process occurring during collisions or encounters between the enzyme and substrate diffusing within

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the lipid bilayer. The second hypothesis takes catalysis as an intramolecular process between a physically associated enzyme and substrate. Models based on these hypotheses make different predictions concerning the statistical and kinetic nature of channel modulation in cell-free patches. Our results indicate that dephosphorylation is an intermolecular process, with the protein phosphatase and the channel existing as separate molecular entities. In contrast, phosphorylation appears to be intramolecular, with the protein kinase and channel existing in some form of stable association.

MATERIALS AND METHODS

Pituitary slices

Thin slices 70 μm thick were cut with a vibratome from freshly dissected rat posterior pituitary as described previously (Jackson et al, 1991; Jackson, 1993). The slices were used immediately for recording or stored for up to two hours. Recordings were made while perfusing slices with a bathing solution containing 125 mM NaCl, 4 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM glucose, bubbled with a mixture of 95% O_2 and 5% CO_2. This same solution was used during the dissection and slicing of the pituitary as well as during short-term storage of slices. Dissection and slicing were carried out at 0°C; recording and storage were at room temperature (21–24°C). Patch clamp recordings were made from round structures measuring up to 15 μm in diameter, which can be seen near the surface of slices under Nomarski optics with a 40X long-working-distance, water immersion objective. These structures have previously been identified by neurobiotin filling techniques as nerve terminals appended to axons (Jackson, 1993).

Patch clamp recordings

Cell-attached patches were formed by sealing patch pipettes against the nerve terminal membrane. After patch excision to form inside-out patches (Horn and Patlak, 1980; Hamill et al., 1981), single channel current was recorded using an EPC-9 patch clamp amplifier (Instrutech, Elmont, NY) interfaced to an Atari computer. Patch electrodes were prepared from borosilicate glass capillaries with an outer diameter of 1.7 mm and an inner diameter of 1.1 mm, coated with sylgard, and filled with 140 mM KCl, 2 mM $MgCl_2$, 0.5 mM EGTA and 10 mM HEPES, pH 7.2. Immediately before excision, the bathing solution was switched to 150 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, 0.5 mM EGTA, pH 7.2. The concentration of free calcium was adjusted by adding $CaCl_2$. The free concentration of divalent cations was calculated with software based on Marks and Maxfield (1991). If not stated explicitly, the computed concentration of free Ca^{2+} at the cytoplasmic membrane face was 250 nM. MgATP was added directly into the recording chamber using a micropipette to achieve a concentration of 1 mM. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Analysis of raw channel data was carried out with the programs REVIEW and TAC (Instrutech). The single channel open probability was determined from records at least 0.3 s long by dividing the area under the channel currents by the number of channels in the patch. The program COSTAT (Cohort Software, Berkeley, CA) was used for statistical analysis. Curve fitting was performed with the program MATHCAD (Mathsoft, Cambridge, MA), by using the MINERR command within a SOLVE block. Means were presented ± SEM.

RESULTS

BK channel density in excised patches

Our examination of enzyme-substrate interactions depended in part on a quantitative assessment of channel density and distribution. We could estimate the number of channels in a patch during strong depolarizations in 250 nM free calcium to increase the single channel open probability to near one. The number of BK channels in an excised patch could then be estimated from the maximum membrane current (Fig. 1 *A*). The number of channels was thus estimated in 163 patches to produce the distribution shown in Fig. 1 *B*. The

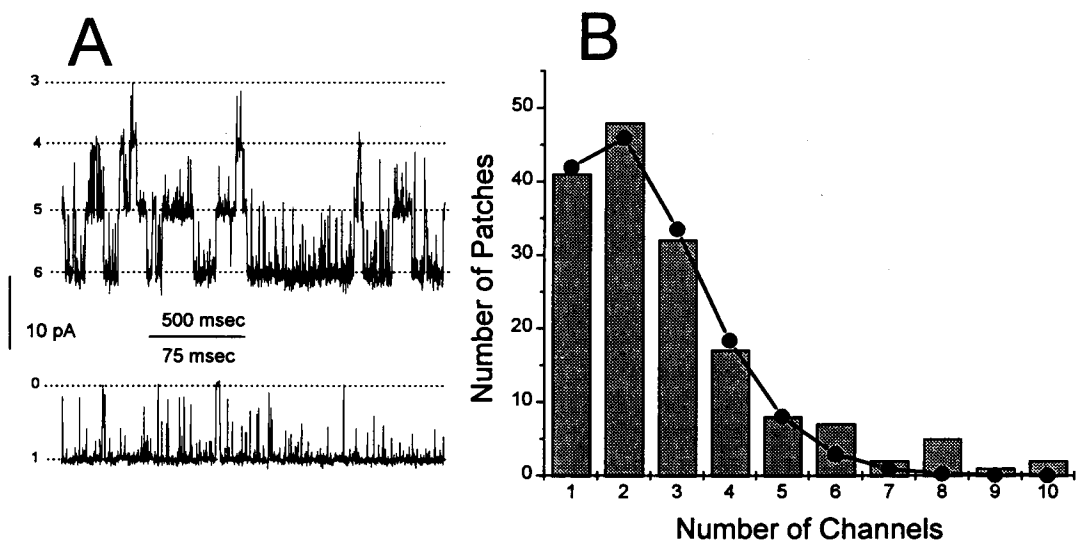

FIGURE 1 (*A*) Single channel current traces recorded before rundown in excised patches containing different numbers of channels. The upper trace was recorded from a patch containing six channels in which the lowest current level shown was the current flowing through all six open channels. Each current level is indicated by a dotted line with the corresponding number of open channels at a given current level indicated by the integers on the left side. The lower trace was recorded from a patch containing only one channel in which the lowest current level is the open single channel current. Patches were held at 30 and 40 mV, respectively, with 250 nM free Ca^{2+} at the cytoplasmic face. The high open probability under these conditions assures that the number of channels can be reliably determined. (*B*) Histogram of channel number in excised patches. The number of channels was estimated in 163 different patches to construct the distribution represented by the bars. The points are the computed best-fitting Poisson distribution (Eq. 1), with the mean number of channels per patch estimated as 2.2, and the total number of patches estimated as 171 (see text).

observed distribution was fitted to the Poisson distribution

$$NP(i) = \frac{N \cdot e^{-m} \cdot m^i}{i!}, \quad (1)$$

in which $P(i)$ is the predicted probability of obtaining a patch with i channels, N is the total number of patches, and m is the mean number of channels per patch. (When these experiments were made, the patches with no BK channels were not noted, so we have no point at $i = 0$, and we must treat the total number of patches, N, as a free parameter.) Fitting Eq. 1 to the data in Fig. 1 *B* yielded a mean number of $m = 2.2$ channels per patch, and a total number of patches $N = 171$.

Visual examination of Fig. 1 *B* would appear to support the idea that channels are distributed randomly among patches according to the Poisson distribution. The quality of the fit can be evaluated by computing χ^2 as

$$\chi^2 = \sum_i \frac{(N_i - NP(i))^2}{NP(i)}, \quad (2)$$

where N_i is the observed number of patches containing i channels, and $NP(i)$ is the expected number of patches with i channels for the best fitting parameters m and N. In this estimate of χ^2, we have exploited the property that the variance of the Poisson distribution is equal to $NP(i)$ (Bevington and Robinson, 1992). The reduced χ^2 for this fit was 41, which clearly negates this model. Thus, despite the appearance of a good fit in Fig. 1 *B*, the distribution of BK channels in membrane patches is not entirely random. The deviation from randomness is primarily for $i = 8$, 9, and 10, i.e., for larger values of i. If we only use i between 1 and 7, a reduced χ^2 of 1.03 ($p > 0.40$) indicates consistency with the Poisson distribution. The estimated mean number of BK channels per patch remained at 2.2 when the data range was thus limited. Thus, BK channels are occasionally found in large clusters, but are otherwise distributed randomly in the membrane. In patches containing seven channels or less, most of the BK channels are probably dispersed in the plasma membrane.

Assuming a nominal patch area of 3 μm^2 (taking a 1-μm radius based on the resistance of our patch pipettes (Sakmann and Neher, 1983)), we obtain a value of 0.7/μm^2 for the density of channels in the membrane. From our previous data (Bielefeldt et al., 1992), we can estimate the macroscopic conductance density through these channels in whole-terminal experiments as 67 pS/μm^2 (activated by voltage steps from −110 to 50 mV). Dividing by the single channel conductance measured in cell-attached patches (Bielefeldt et al., 1992), we obtained a channel density of 0.5/μm^2, which compares well with the above estimate of channel density from experiments in excised patches.

Rundown of channel activity

When inside-out patches containing BK channels were excised in a solution with no MgATP, the channel activity declined abruptly after a variable delay (Bielefeldt and Jackson, 1994). This is illustrated in plots of single channel open probability versus time after excision (Fig. 2). Rundown can thus be viewed at the molecular level as a discrete process, reflecting a switch between two gating modes concomitant with a catalytic event. This was especially clear in patches containing only one channel (Fig. 2). We have previously shown that the protein phosphatase inhibitor okadaic acid blocks this rundown, and for this and other reasons we concluded that an enzymatic dephosphorylation causes the decline in channel activity (Bielefeldt and Jackson, 1994). This rundown of channel activity was manifest as a reduction in single-channel open probability from approximately 0.9 to 0.1 in patches held at −40 mV in 250 nM Ca^{2+}.

Rundown was completed within 5 min after excision in 136 of 142 (95.8%) patches. All of these 142 patches lasted 5 min or more without breaking. In the six patches showing no rundown, channel activity changed little. Of these 142 patches, 2 lasted for 18 and 35 min, respectively, with channel activity changing by less than 20%. The probability of these outcomes is considered below.

Restoration of channel activity by ATP

After rundown, adding MgATP to the recording chamber restored BK channel activity (Bielefeldt and Jackson, 1994). The MgATP added in this way has access to the cytoplasmic face of excised patches. This effect of ATP depended on magnesium, could not be supported by a nonhydrolyzable ATP analog, and could be blocked by the protein kinase inhibitors staurosporine and 1-(5-isoquinolinylsulfonyl)-3-methylpiperazine. These results indicated that a membrane-bound protein kinase mediated the MgATP-induced recovery (Bielefeldt and Jackson, 1994). As with the protein phosphatase, a high proportion of patches containing a BK

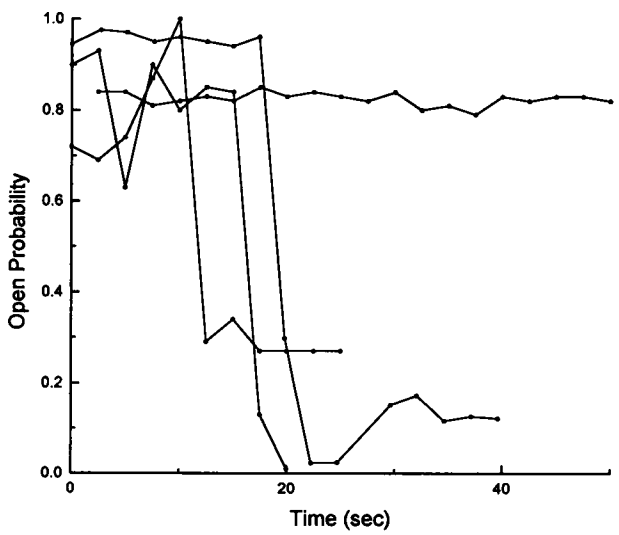

FIGURE 2 Rundown in patches containing one channel. P_o was determined at regular 2.5-s intervals in records from excised patches. Measurement began at the time of patch excision. In three of the four plots, the channel activity dropped abruptly at variable times after excision, thus marking the moment of dephosphorylation. No rundown occurred during the time shown in a fourth plot.

channel also contained functional protein kinase: MgATP restored channel activity in 113 out of 115 (98.3%) patches. The probability of these outcomes is considered next.

Probability of enzyme-free patches

Intermolecular case

As just noted, patches containing a BK channel only rarely lacked protein phosphatase or protein kinase activity. If the enzyme and channel are separate molecules such that catalysis is intermolecular, then one can use the first term of the Poisson distribution (Eq. 1) to estimate the probability of a patch lacking one of these enzymes:

$$P(0) = \exp(-m) = \exp(-A \cdot \rho), \quad (3)$$

where m = mean number of enzymes per patch, A = patch area, and ρ = number of enzymes per unit area of membrane. With 4.2% of the patches containing no protein phosphatase, we take $P(0) = 0.042$, and obtain a value for m of 3.2 molecules of protein phosphatase per patch. With 1.7% of the patches containing no protein kinase, we take $P(0) = 0.017$ and obtain a value for m of 4.1 molecules of protein kinase per patch. Assuming a nominal patch area of 3 μm^2 (as explained above during the estimation of channel density) gives $\rho = 1\ \mu m^{-2}$ and $1.4\ \mu m^{-2}$ for the membrane densities of protein phosphatase and protein kinase, respectively.

The above calculations were based on the assumption that patches failing to show rundown or recovery lacked the necessary enzyme. However, consideration of the stochastic nature of processes viewed at the molecular level might support an alternative explanation that, in these patches, the catalytic events had by chance not yet occurred; such long time intervals could lie on the tail of a characteristic waiting time distribution. Analysis of the kinetics of rundown and recovery provides a means of estimating the probability of these reactions not occurring during a particular observation period. The time course of rundown averaged for many patches was well fitted by a single exponential with a time constant of 36.5 s (see below). The probability of rundown not occurring after 5 min is then 0.00027. (Fitting to an alternative model described below yielded a similar very low probability for catalysis failing to occur in 5 min). Thus, if every patch contained active enzyme, then one would have to look at 3700 patches to have a reasonable chance of finding even one to maintain activity for more than 5 min. Obtaining 6 out of 142 is clearly not within the observed distribution of waiting times. Consideration of the two patches in which activity lasted 18 and 35 min without rundown indicates an extremely low probability that these patches contained functional protein phosphatase. Thus, absence of rundown was not a question of degree. Patches either possessed or lacked protein phosphatase activity. We can then conclude that the absence of rundown reflected an absence of functional enzyme in those patches rather than natural fluctuations in waiting times measured at the molecular level. The lower incidence of unsuccessful recovery and the very complicated kinetics of the recovery process (see below) made a similar analysis of the protein kinase more difficult. However, the rapidity of recovery shown below made it unlikely that the two instances in which recovery was not observed were due to insufficient time.

Intramolecular case

To interpret the above results in terms of an intramolecular model for catalysis, we must explain the patches lacking enzymatic activity as either channels dissociated from the relevant enzyme, channels associated with defective enzymes, or channels in some way resistant to dephosphorylation. The present methods of analysis make no distinction between these three possibilities. In the case of an association equilibrium between the enzyme and channel, the concentration ratio of enzyme-free to enzyme-bound channels is given by

$$\frac{[\mathrm{BK}]}{[\mathrm{BK} \cdot \mathrm{E}]} = \frac{K_d}{[\mathrm{E}]}, \quad (4)$$

where the symbols for concentrations of BK channel, BK channel-enzyme complex, and enzyme are self-explanatory, but concentration is in units of number per unit of membrane area. K_d is a dissociation constant in similar units for enzyme-channel complex formation. With the six patches showing no rundown containing nine channels, and the 136 patches showing rundown containing 299 channels, this ratio for protein phosphatase is then 33. The same calculation for protein kinase shows that this enzyme is present in a 125-fold excess over its dissociation constant with the channel. Considered from the perspective of the intramolecular model, these ratios imply either strong associations or high membrane densities of enzyme.

Correlations between enzyme number and channel number

The experiments mentioned above in which no rundown was seen for observation periods of more than 5 min provided additional information relevant to the question of intra- versus intermolecular mechanisms. Of the six patches showing no rundown, three of these contained one BK channel, and three contained two BK channels. Thus, three patches with one channel and three patches with two channels contained no functional protein phosphatase. However, if the enzyme and channel are parts of a complex, in which lack of rundown reflects absent or defective protein phosphatase, then the probability of obtaining enzyme-free patches should decrease with the number of channels per patch. Because rundown is seen in such a high proportion of patches, we can presume that if channels form complexes with enzymes, the enzymes are rarely absent or defective. The probability of a patch containing two channels, both of which coincidentally lack enzyme, should then be much lower than the probability of a patch containing only one channel without functional enzyme. The finding that the same number of patches with two channels as with one channel failed to show rundown

therefore casts doubt on the intramolecular model in the case of dephosphorylation.

A statistical analysis provided a more critical test of this interpretation. According to the intramolecular hypothesis, absence of rundown will be seen most often in patches with only one BK channel. The probability of a patch with N channels all failing to exhibit rundown should go as b^N, where b is the probability of a single enzyme-channel complex lacking enzyme activity. Using the Poisson distribution for the probability of i channels in a patch (Eq. 1), we expect the following expression to give the number of patches with i channels, all of which fail to show rundown:

$$K(i) = \frac{N \cdot e^{-m} \cdot m^i}{i!} \cdot b^i. \quad (5)$$

Rundown was studied in $N = 142$ patches; $m = 2.2$ was determined from the fit of Eq. 1 to the distribution shown in Fig. 1 *B*. The single remaining parameter b can then be estimated by fitting Eq. 5 to the data points, namely $K(1) = 3$, $K(2) = 3$, and $K(i) = 0$ for $i > 2$ (Fig. 3). This fit yielded $b = 0.103$. Computing χ^2 in a fashion similar to Eq. 2 gave a reduced $\chi^2 = 1.68$ ($p < 0.1$). However, for larger values of i both theory and experiment give $K(i)$ near zero, so that this χ^2 based on all 10 points is not very sensitive to a poor fit. Limiting the fit to $i \leq 5$ gives the same value for b, indicating that the excluded data has little discriminating value. But using a χ^2 reduced by 5 points instead of 10 gave $p < 0.01$. Although the χ^2 test is of questionable validity when the bins have so few elements, we can gain additional insight by focusing on the point $K(3) = 2$ and using the best-fitting parameters and Eq. 5 to estimate the probability of obtaining a patch containing two channels and no enzyme. The predicted mean and variance are both 0.4, and the probability of obtaining the observed number of 3 patches out of 142 is 0.007. This analysis confirms the χ^2 test in rejecting the intramolecular model based on the observed distribution of channels in patches showing no rundown.

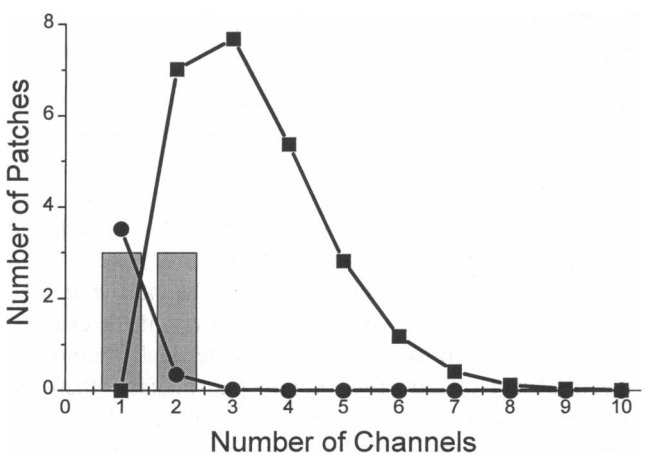

FIGURE 3 Histogram of channel number in patches showing no rundown. Bars denote the number of patches with a given number of channels in which no rundown occurred. The circles show the prediction based on the intramolecular model (Eq. 5). Because $\chi^2 = 3.36$ for $i \leq 5$ ($p < 0.01$), the intramolecular model is inconsistent with these results. The squares show the prediction by the intramolecular model of the number of patches expected to show partial incomplete rundown (Eq. 6). Because no patches showed partial incomplete rundown, this result also was inconsistent with the intramolecular model.

Evaluating the probability of patches showing partial but incomplete rundown provided additional support for this conclusion. Rundown to a final intermediate level of channel activity was never observed among the 142 patches that lasted more than 5 min. According to the intramolecular model, in patches with many channels one expects rarely to encounter extreme cases of complete rundown or complete absence of rundown. In contrast, patches showing partial rundown should be more likely in patches with many channels. The binomial distribution can be used to determine the probability of finding a patch with i channels in which one or more, but not all, enzyme-channel complexes lack associated enzyme activity. This probability is $1 - (1 - b)^i - b^i$, which when combined with the Poisson distribution, gives the following expression for the number of patches expected to have i channels and show partial incomplete rundown:

$$J(i) = \frac{N \cdot e^{-m} \cdot m^i}{i!} \cdot (1 - (1 - b)^i - b^i). \quad (6)$$

This expression is plotted in Fig. 3. Clearly, a substantial number of patches showing partial rundown were expected. The fact the none were seen supported the conclusion that the channel and protein phosphatase are not parts of a stable complex.

These considerations indicate that the absence of rundown in some patches cannot be explained by absent or defective protein phosphatase within a stable enzyme-channel complex. The absence of rundown also cannot be explained as occasional resistance of channels to dephosphorylation. Thus, an analysis of the correlation between channel number and enzyme number favors the model of channel and protein phosphatase as separate molecules in which the catalytic event is intermolecular.

The lower incidence of patches showing no recovery made it more difficult to evaluate channel-protein kinase associations by the same technique. Each of the two patches in which channel activity could not be restored had only one channel. This is qualitatively what one expects for the intramolecular model (*circles* in Fig. 3), but the lower incidence of lack of recovery made a quantitative statistical argument more difficult. The lower incidence of patches containing at least one BK channel and lacking functional protein kinase can be taken as weak evidence in favor of the hypothesis of protein phosphorylation as an intramolecular process between tightly associated channel and protein kinase.

Time course of rundown

The kinetics of rundown and recovery can be investigated to provide additional insight into the nature of enzyme-channel interactions. However, in a typical patch with only one or two channels, rundown and recovery appeared as nearly instan-

taneous jumps, with latencies reflecting the stochastic nature of the underlying molecular process (Fig. 2). In a patch with one channel, one patch excision experiment yields a measurement of a single time. Each measurement of a time until rundown or recovery is equivalent to the measurement of a conductance state dwell time in single-channel kinetics (Jackson, 1992). Because one needs many such lifetime measurements to evaluate the time course of these reactions, we averaged the results from 35 experiments performed under identical conditions (Fig. 4). In each experiment, a patch was excised into MgATP-free solution and the open probability was measured at 2.5-s intervals (Fig. 2). Subsequently, we normalized the channel activity to the maximum value, and averaged this normalized current across patches at each time point. For the simplest type of intramolecular process, we expect the time course of average current to be well fitted by a single exponential function of the form

$$I = A \cdot e^{-t/\tau} + B. \tag{7}$$

Fitting this expression to the data of Fig. 4 yielded a time constant of $\tau = 36.5$ s, and values for the other parameters of $A = 0.70$ and $B = 0.22$. Note that the parameter B is necessary for two reasons: 1) After rundown one sees a low but stable probability of channel opening (Bielefeldt and Jackson, 1994), and 2) as discussed above, a small number of patches showed no rundown.

In contrast to the intramolecular case, catalysis as an intermolecular process predicts a dispersion in rundown rates reflecting the variable number of protein phosphatase molecules per excised patch. We have already used the Poisson distribution to estimate the mean number of protein phosphatase molecules per patch from the number of protein phosphatase-free patches. We can also use this distribution to predict the time course for rundown of current according to the intermolecular model

$$I = A \cdot \left(\sum_i \frac{e^{-m} \cdot e^{-i \cdot t/\tau} m^i}{i!} \right) + B. \tag{8}$$

This expression follows from the assumption of a Poisson distribution in the number of enzymes in a patch, with the rate of dephosphorylation taken as a fundamental value, $1/\tau$, times the number of molecules of enzyme, i, per patch. Equation 8 (with the sum truncated to 20 terms) was fitted to the data of Fig. 4, yielding $A = 0.83$, $B = 0.11$, $\tau = 98.5$ s, and $m = 2.6$.

Fitting to Eq. 8 yielded an RMS error of 0.013 that was only slightly better than the RMS error of 0.016 obtained from the fit to Eq. 7. On the basis of quality of fit, the models represented by Eqs. 7 and 8 must be viewed as indistinguishable. But consideration of the value obtained for m in the fit of Eq. 8 provides additional evidence in favor of the intermolecular model. This fit produced $m = 2.6$ as the mean number of protein phosphatase molecules per patch, and the above Poisson analysis of the number of patches showing no rundown gave a value of $m = 3.2$. Similar estimates of m obtained from analysis of different experimental results provided a strong test for consistency with the intermolecular model.

We examined the reproducibility of the rundown rate by cycling patches through two successive rundown experiments under identical conditions. After rundown was complete, MgATP was added to restore activity and the process was repeated. In 13 patches, channel activity was normalized and averaged as described above. The averaged membrane current of the first experiment followed a time course similar to the averaged membrane current of the second experiment (Fig. 5 *A*).

We have previously shown that voltage enhances restoration of channel activity by phosphorylation (Bielefeldt and Jackson, 1994). However, we found no such effect of voltage on rundown. When we held 20 inside-out patches at 40 mV, channel activity declined with the same rate as it did at −40 mV (Fig. 5 *B*). Because two-dimensional diffusion in the plane of the membrane is expected not to depend on voltage, this result is consistent with rundown having as a rate-limiting step collisions between two diffusing species. Thus, rundown was reproducible, proceeding with the same voltage-independent rate in repeated trials.

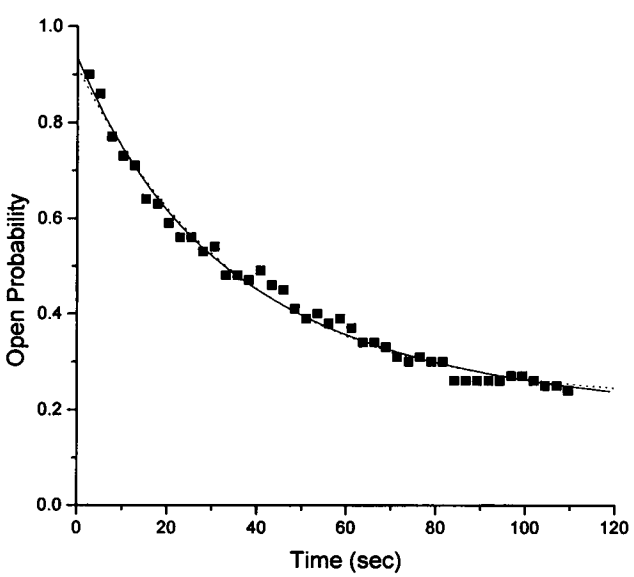

FIGURE 4 Traces from 35 experiments such as those shown in Fig. 2 were averaged to construct a macroscopic plot of rundown kinetics. This average trace was fitted to Eq. 7 (· · · · ·) and to Eq. 8. (——). Both curves are drawn; they run through the data and virtually overlie one another except at the very beginning and end of the plot. Results of the curve fits are given in the text.

Time course of restoration

Restoration is much more rapid and much more complicated than rundown, so an analysis of restoration kinetics similar to that presented above for rundown kinetics could not be performed. This complexity of restoration kinetics was reflected in the time course with which channel activity returned following MgATP addition to the recording chamber after allowing channel activity to rundown (Fig. 6). When the

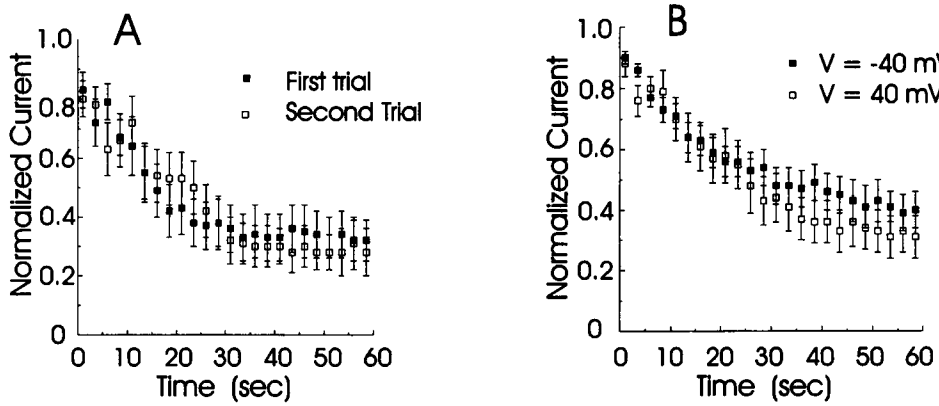

FIGURE 5 (*A*) Comparison of rundown kinetics before and after cycling through rundown and restoration. In 13 experiments, the first occurrence of rundown (■) was compared with the second (□), determined under identical conditions. After the first occurrence of rundown, activity was restored to initial levels by adding MgATP. MgATP was then removed to allow rundown to occur again. (*B*) The kinetics of rundown was compared at −40 mV (■) and 40 mV (□) in 20 patches. Plots were constructed by averaging over patches for a given time after excision and normalizing to the initial value.

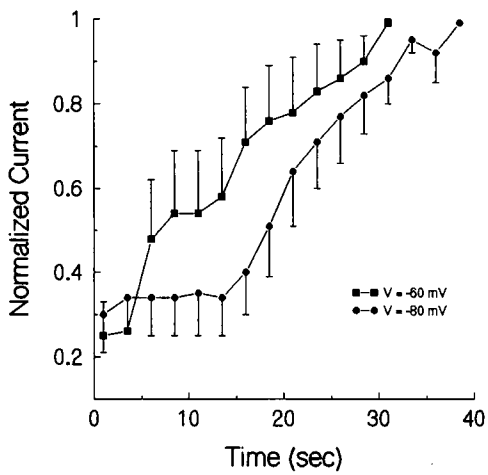

FIGURE 6 Time course of restoration. Inside-out patches were excised into a solution that contained no MgATP. After rundown of channel activity was complete, 1 mM MgATP was added by pipette to the recording chamber, while patches were held at −80 mV (●; $n = 8$) or −60 mV (■; $n = 8$). At −60 mV, activity recovered more rapidly than at −80 mV ($p < 0.01$ from ANOVA). The membrane current after addition of MgATP was normalized to the maximal current during the experiment and averaged over all patches for a given voltage.

holding potential was kept at −80 mV, channel activity increased after a delay of several seconds. We normalized current to the maximal value reached after the addition of MgATP, and averaged the results across patches from eight experiments. In contrast to the monotonic decay of channel activity during rundown (Figs. 4 and 5), restoration proceeded after a delay that varied with membrane potential. Increasing voltage to −60 mV reduced the time to recovery (Fig. 6). The more rapid time course seen at −60 mV indicated that the delay in onset of restoration at −80 mV did not reflect the speed of mixing of MgATP in the recording chamber.

Because the technique employed above of adding ATP to the bath by pipette was too slow to study the kinetics of restoration of channel activity at potentials more positive than −60 mV, we took advantage of the slower rate of recovery at hyperpolarized potentials to add MgATP. This provided sufficient time for mixing before initiating phosphorylation with a step to a more positive potential. Because phosphorylated pituitary BK channels open within a few milliseconds after voltage steps (Bielefeldt et al., 1992; Bielefeldt and Jackson, 1993), the observed increase in channel activity reflected phosphorylation. With this method, we could measure more rapid rates of restoration than by pipette addition of ATP. Time to recover was measured in response to voltage steps and plotted versus voltage (Fig. 7). This plot, which included data from 40 experiments performed in 21 patches, confirmed the dependence of the phosphorylation

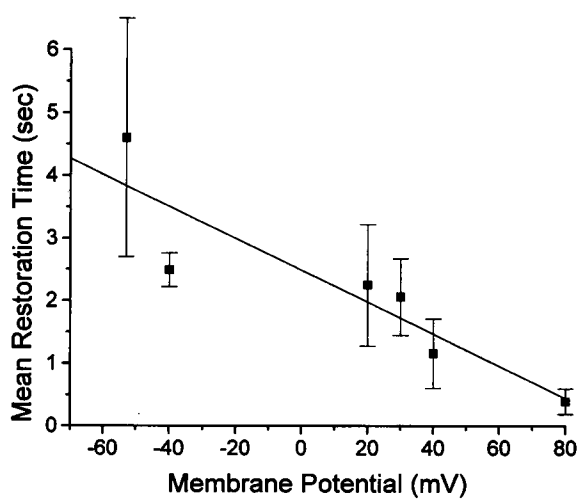

FIGURE 7 Restoration time versus voltage. After channel rundown, the patches were kept at a holding potential of −80 mV. MgATP (1 mM) was added, and restoration of channel activity was then initiated by depolarizing steps from −80 mV to various test potentials. The mean time for return of channel activity was then plotted against the test potential. In patches with two or more channels the time for return of activity was taken as the time for P_0 to rise to 50% of its final mean value. The best fitting line was drawn. The significance level obtained from linear regression analysis was $p < 0.02$.

rate on voltage (Bielefeldt and Jackson, 1994) and extended the range over which this dependence can be demonstrated.

We used the technique of voltage steps to study the kinetics of restoration in two patches that contained eight BK channels. The large number of channels present in these patches allowed us to measure restoration as a quasi-macroscopic process. As in the above experiment (Fig. 7), after rundown was complete, ATP was added at a negative voltage to allow mixing before channel activity was restored. Stepping the patch to a positive voltage then induced phosphorylation. Fig. 8 *A* shows two such experiments with voltage steps to 10 and 30 mV. The final current amplitudes were scaled to the same level to compare the time course of recovery under different conditions. These records confirm the sigmoidal time course displayed in Fig. 6. The experiments in Fig. 6 were all performed with patches having less than seven channels. Recall that the analysis of the channel distribution presented above (Fig. 1) suggested that patches with eight or more channels result from clustering of channels, whereas in patches with a lower density, the channels were probably dispersed. Because two patches with eight channels exhibited sigmoidal kinetics similar to that seen in patches with fewer channels, it is unlikely that the clustering of channels strongly influenced the kinetics of phosphorylation. This is especially significant because sigmoidal kinetics such as shown in Figs. 6 and 8 are often taken as a sign of cooperativity. The similar results from patches with many and few channels suggest that the mechanism underlying the sigmoidal time course of phosphorylation does not involve cooperative interactions between channels.

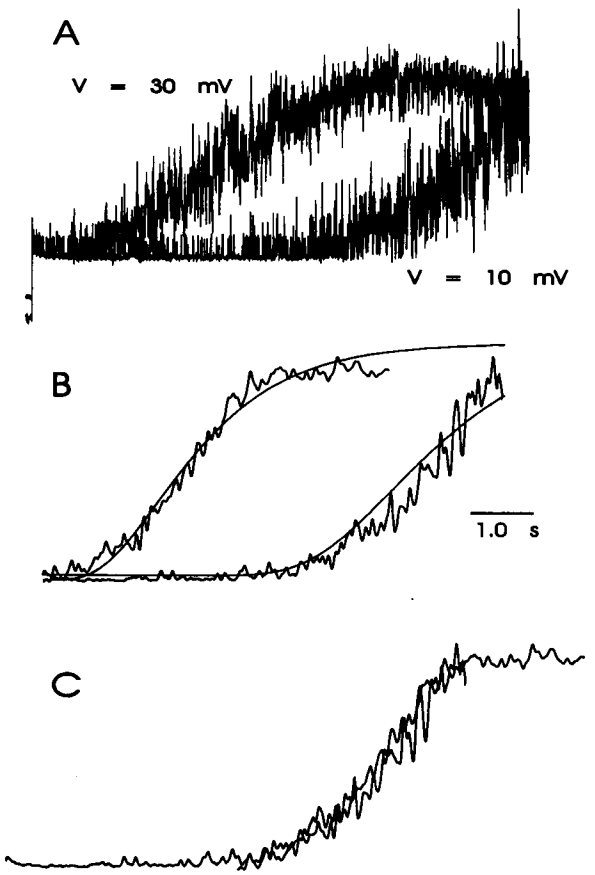

FIGURE 8 In a patch with 8 BK channels, current had a quasi-macroscopic character. After channel rundown, 1 mM MgATP was added and the patch was immediately stepped from −80 mV to 10 or 30 mV. (*A*) Unfiltered traces show the time course of restoration of channel activity with single channel levels visible. (*B*) The traces were filtered at 5 Hz to reduce the channel noise, and then fitted to Eq. 9 to give $\tau = 1.5$ and $n = 61$ for 10 mV ($R^2 = 0.96$), and $\tau = 1.2$ and $n = 5.1$ for 30 mV ($R^2 = 0.96$). (*C*) The two traces shown in B were displaced relative to one another so that the regions in which channel activity increased most steeply were superimposed. This suggests that voltage shortens the delay without increasing the steepness.

The current records in Fig. 8 *A* were digitally filtered at 5 Hz to reveal the average behavior more clearly (Fig. 8 *B*). This showed that voltage influences the delay before channel activity increases rather than the slope of the increase, which confirms another qualitative aspect of phosphorylation kinetics observed in patches with fewer channels (Fig. 6). The steepness of the rise in channel activity appeared not to vary with voltage. The filtered traces could be well fitted by an exponential raised to a power:

$$I = I_{min} + (I_{max} - I_{min})1 - e^{-t/\tau})^n. \quad (9)$$

For both 10 and 30 mV, the time constants needed to fit these time courses were similar, but the exponents to which the exponentials were raised differed by a factor of 12 (Fig. 8 *B*, *legend*). The voltage dependence of the exponents, together with their large magnitudes, defy an explanation in terms of cooperativity between protein subunits. A simple explanation of n as the number of phosphates attached to the protein to activate the channel is also unlikely in view of the large changes in n. It is more likely that this voltage-dependent delay reflects a distinct kinetic step early in the process. Such a model has been used successfully to explain another variable delay in current activation known as the Cole-Moore shift (Taylor and Bezanilla, 1983). As with the Cole-Moore shift, simple displacement of one trace relative to the other on the time axis makes them nearly superimposable (Fig. 8 *C*). However, in contrast to the present phenomenon in which the delay depends on the present voltage, in the Cole-Moore shift the delay depends on the prior voltage:

The time course of restoration clearly reveals at least two distinct kinetic steps, the first of which depends on voltage. In an intermolecular process, the first step is necessarily diffusion resulting in collisions. Because it is hard to envision a membrane protein with a diffusion constant so strongly dependent on membrane potential, if restoration of channel activity is intermolecular, the collision must precede even the initial step responsible for the observed delay. This would impose the requirement that the collision step be significantly faster than the total rate measured for recovery. As will be shown below, this rate exceeds the diffusion limit.

Diffusion-limited catalysis

If an enzymatic process in a membrane depends on random collisions between two proteins, its rate should not exceed a

theoretical limit prescribed by two-dimensional diffusion in lipid bilayers. Mathematical models have been developed employing the theory of mean-first-passage times (Lifson and Jackson, 1961) to describe diffusion-limited kinetic processes in membranes (Berg and Purcell, 1977; Wofsy and Goldstein, 1984). These studies suggest the following expression for the mean time, $\bar{t}$, for a small diffusing particle to be captured by a perfectly absorbing circular target of radius r in the center of a larger circular domain with radius σ.

$$\bar{t} = \frac{\sigma^2}{2D}\left[\ln\left(\frac{\sigma}{r}\right) - \frac{3}{4}\right]. \quad (10)$$

This is an average time over all possible initial positions of the diffusing particle anywhere within the circle outside of the target. In this case the circular domain is the excised membrane patch, and the target is the BK channel. D denotes the two-dimensional diffusion coefficient. Given the geometry of an excised patch, this model is highly appropriate for the present experiments, except for the minor restriction that the target lies in the center of the circle.

We will assume a value of 1 μm for σ, the radius of the patch of membrane, as discussed above (Sakmann and Neher, 1983). Examination of models based on the primary sequence of the voltage-gated sodium channel (Guy and Conti, 1990) suggests that the radius is within 2.5 Å of 32.5 Å (R. Guy, personal communication). Because a calcium-activated potassium channel has a similar primary structure (Atkinson et al., 1991), we will use this value for r. The diffusion constant in Eq. 10 is more generally the sum of the diffusion constants of the two proteins. The diffusion constant of the BK channel is probably no greater than that of the unanchored sodium channel, which is on the order of 10^{-9} $cm^2\ s^{-1}$ (Angelides et al., 1989). However, the enzyme is likely to be a peripheral membrane protein with a higher diffusion constant. After considering similar issues, Saxton and Owicki (1989), settled on a value of 10^{-8} $cm^2\ s^{-1}$ for transducin. We will use this value here and neglect the small increment from the addition of the smaller diffusion constant of the channel. With these choices for σ, D, and r, the predicted mean time from Eq. 10 of the maximum rate of encounters between the channel and the enzyme is $\bar{t}$ = 2.5 s.

Our best estimate of the mean time for dephosphorylation according to the intermolecular model was obtained by fitting Eq. 8 to give τ = 98 s (Fig. 4). The rate of dephosphorylation can be accelerated approximately fivefold by activation of a G-protein (Bielefeldt and Jackson, 1994) to yield a lower bound to the mean time for dephosphorylation of 20 s. With the above estimate of mean number of protein phosphatase molecules per patch of approximately 3, we obtain a mean time for rundown of 60 s per molecule of protein phosphatase. This is nearly 24-fold slower than the diffusion limited computed above. Thus, we can conclude that the observed rate of rundown is slow enough to be diffusion limited. It is thus consistent with the other results presented above that suggest that dephosphorylation is intermolecular.

In contrast to the relatively slow rate of rundown, channel restoration at positive voltages proceeded more rapidly than the diffusion limit. A depolarizing pulse to 80 mV induced recovery with an average time of 396 ms (Fig. 7). With the diffusion limit of 2.5 s computed above for a patch with only one molecule of enzyme, and the Poisson estimate for the mean number of enzymes per patch of 4.1, we can estimate the fastest time for phosphorylation as an intermolecular process to be 610 ms. This makes the observed mean restoration time of 396 ms at 80 mV too fast to be diffusion limited, and the gap grows under the following considerations. Because the voltage-dependent step occurs before the actual increase in channel activity (Figs. 6 and 8), and the diffusion-dependent collision step would have to precede that, we can conclude that the putative collision step would have to take place in less than the observed restoration time. Likewise, because Eq. 10 is based on the assumption that all collisions, irrespective of orientation and point of contact result in catalysis, it is a very high estimate of the diffusion limited rate. To the extent that we can make a reliable estimate of the theoretical diffusion limit, restoration appears to be too fast to be intermolecular. This analysis supports the hypothesis that phosphorylation is intramolecular, with the channel and protein kinase residing together within a macromolecular complex.

DISCUSSION

This study has shown how kinetic and statistical data from excised patches can be used to distinguish between intramolecular and intermolecular mechanisms in the enzymatic reactions that modulate the activity of ion channels. The dephosphorylation reaction behaves as an intermolecular process resulting from collisions between the enzyme and its substrate. This conclusion is supported by the following observations. 1) The number of enzyme molecules per patch estimated from the number of enzyme-free patches and from the kinetics of rundown were in close agreement. 2) The probability of enzyme-free patches did not decrease with increasing number of channels per patch. 3) There were no instances of partial incomplete rundown, even in patches containing two or more channels. 4) The rate of rundown was slower than the diffusion limit. In contrast to dephosphorylation, the phosphorylation reaction behaved more as an intramolecular process. 1) Protein kinase-free patches were less frequently observed. 2) The rate of restoration exceeded the diffusion limit. Thus, both intra and intermolecular mechanisms operate within the membranes of posterior pituitary nerve terminals.

The association of enzymes with membranes brings the enzymes closer to their membrane bound substrates. However, the kinetic advantage of this arrangement as a reduction in dimensionality has been questioned (McCloskey and Poo, 1986). Clearly, a kinetic advantage is afforded by the apparent association between the BK channel and a protein kinase, allowing the reaction to proceed at a rate faster than that allowed by diffusion. This offers a considerable advan-

tage in permitting rapid transduction of signals to alter membrane excitability, although an enzyme thus dedicated would suffer a restriction in function to the modification of a single molecule of substrate. Chung et al. (1991) observed a similar association between a BK channel and a protein kinase purified from rat brain and reconstituted into an artificial lipid bilayer. The persistence of enzymatic activity after removing the channel from its native environment and reconstituting into artificial lipid bilayers argues in favor of a close association between the channel and enzyme, as does the rate of phosphorylation exceeding the diffusion limit. We have previously pointed out a number of other similarities between a channel and protein kinase studied in Levitan's laboratory (Reinhart et al., 1991) and those we are studying in the posterior pituitary (Bielefeldt and Jackson, 1994). The evidence for association between the channel and protein kinase presented here thus strengthens the case for identity between the molecules studied in our laboratory and Levitan's. The identity and molecular characteristics of this channel have added physiological relevance because in posterior pituitary nerve terminals this BK channel has the capacity to reduce action potential threshold in a manner consistent with a function in use-dependent depression of secretion (Bielefeldt and Jackson, 1993, 1994).

Although experiments with excised patches and reconstituted ion channels suggest a physical link between the channel protein and modulating enzymes, the results of biochemical and molecular biological studies go even further, and question the distinction between ion channel and enzyme. Rehm et al. (1989) demonstrated that a purified potassium channel exhibited protein kinase activity, which could not be separated from the purified channel protein by conventional biochemical techniques. This could indicate that the channel protein itself contains an enzymatically active domain. The molecular characterization of ion channels has provided additional data to support this concept. The deduced primary structures of several ion channels contain consensus sequences for ATP-binding sites (Riordan et al., 1989; Atkinson et al., 1991; Thomas et al., 1991; Jan and Jan, 1992; Ho et al., 1993). Functional assays of expressed ion channels showing modulation by ATP confirmed the relevance of this structural homology with protein kinases (Thomas et al., 1991; Ho et al., 1993).

It is interesting to view the two models tested in the present study within a spectrum of possible interactions that can exist between modulating enzymes and their membrane-associated substrates. One can envision at one end of the spectrum a water-soluble enzyme that encounters the membrane protein substrate from the aqueous milieu to form a transient catalytic complex. The next level is the intermolecular case examined here, in which the enzyme is membrane-bound, but still must diffuse to encounter its substrate from the membrane milieu. A third level of interaction would then be two distinct polypeptides forming a stable complex within the membrane so that no binding step is necessary. Finally, the most intimate association is achieved when the polypeptides that form the channel also comprise the enzyme. Each of these forms of interaction between enzyme and substrate offers distinct possibilities in the regulation of membrane function.

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