Modification of Potassium Channel Kinetics by Amino Group Reagents

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ABSTRACT We have examined the actions of several amino group reagents on delayed rectifier potassium channels in squid giant axons. Three general classes of reagents were used: (1) those that preserved the positive charge of amino groups; (2) those that neutralized the charge; and (3) those that replace the positive with a negative charge. All three types of reagents produced qualitatively similar effects on K channel properties. Trinitrobenzene sulfonic acid (TNBS) neutralizes the peptide terminal amino groups and the ε-amino group of lysine groups. TNBS (a) slowed the kinetics of macroscopic ionic currents; (b) increased the size of ionic currents at large positive voltages; (c) shifted the voltage-dependent probability of channel opening to more positive potentials but had no effect on the voltage sensitivity; and (d) altered several properties of K channel gating currents. The actions of TNBS on gating currents suggest the presence of multiple gating current components. These effects are not all coupled, suggesting that several amino groups on the external surface of K channels are important for channel gating. A simple kinetic model that considers the channel to be composed of independent heterologous subunits is consistent with most of the modifications produced by amino group reagents.

INTRODUCTION

Potassium channels serve a variety of roles in excitable tissues. Current through delayed rectifier K channels in axons helps to terminate the action potential. Rapidly inactivating (A-type) K channels regulate repetitive action potential activity in neurons (Connor and Stevens, 1971). Ca2+-activated K channels control bursting behavior in many cell types (Meech and Standen, 1975). Transmitter modulated K channels may underlie simple forms of learning (Alkon, 1979; Siegelbaum et al., 1982).

Full understanding of this large diversity of K channel types requires molecular information on channel sequence, structure, and function. To this end, the amino acid sequences of many inactivating and delayed rectifier K channels have been determined (reviewed in Jan and Jan, 1990). Mutagenesis experiments have yielded important information on the molecular basis of gating of A-type channels (Hoshi et al., 1990; Papazian et al., 1991). Much less is known about the functional role of
specific amino acids in the gating of delayed rectifier channels, but modification of 
histidine residues on the external surface of one such channel produces specific 
changes in activation kinetics (Spires and Begenisich, 1990).

We have examined the functional changes produced in squid axon K channels by 
reagents that react with amino groups. This study included measurements of both K 
channel macroscopic ionic currents and gating currents. We found that amino group 
reagents (a) slowed the kinetics of macroscopic ionic currents; (b) increased the size of ionic currents at large positive voltages; (c) shifted the voltage-dependent probability of channel opening to more positive potentials but had no effect on the voltage sensitivity; and (d) altered several properties of K channel gating currents. The actions of trinitrobenzene sulfonic acid (TNBS) on gating currents suggest the presence of multiple gating current components. We found different rates of modification of some of these channel properties, suggesting that more than one amino group on the external surface of K channels is important for channel gating. We consider the kinetic changes produced by amino group modification in terms of a simple model in which the channel is composed of independent, heterologous subunits.

Preliminary reports of these findings were presented at annual meetings of the Biophysical Society (Spires and Begenisich, 1987; Spires et al., 1988).

METHODS

Biological Preparation

The data in this report were obtained with giant axons from the squid Loligo pealei at the Marine Biological Laboratory, Woods Hole, MA.

Voltage Clamp and Internal Perfusion

The axons used in this study were internally perfused and voltage clamped using techniques that have previously been described in detail (Begenisich and Lynch, 1974; Busath and Begenisch, 1982). Details on our techniques for measurement of K channel gating currents can be found in Spires and Begenisich (1989). All voltages have been corrected for the junction potential between the internal 0.56 M KCl electrode and the internal solutions. External potentials were measured with an agar-filled 3 M KCl electrode. Most of the measurements of ionic currents were done at a temperature of 15°C. Gating currents were measured at 20°C. Series resistance compensation was used.

Membrane currents were measured with a 12-bit analog/digital converter controlled by a microcomputer of our own design or by an IBM PC/XT. The voltage-clamp pulses were generated by a 12-bit digital/analog converter controlled by the computer system. Linear capacitative and leakage currents were subtracted from most records using a \(\pm P/4\) procedure (Bezanilla and Armstrong, 1977). For ionic current measurements, the baseline potential for the P/4 pulses was the holding potential; a baseline potential of about \(-100\) mV was used for gating current measurements. To improve the signal/noise ratio of gating current measurements, 16 or 32 records were averaged.

Solutions

Ionic currents. The external solution used for measurements of ionic currents was a high potassium artificial sea water (50 K ASW) containing (mM) 390 NaCl, 50 KCl, 10 CaCl₂, 50
MgCl₂ and 10 HEPES buffer, pH near 7.4. The elevated potassium was used to reduce the effects of K⁺ accumulation in the periaxonal space associated with these axons (Frankenhaeuser and Hodgkin, 1956). Na⁺ channel currents were blocked by addition of 1 μM tetrodotoxin (TTX). The external solutions had osmolarities of ~975 mosmol/kg. The standard internal solution (K SIS) contained (mM) 50 KF, 270 K glutamate, 15 K₂HPO₄, and 390 glycine, and had a pH of 7.4 and an osmolarity of 970 mosmol/kg.

**Gating currents.** The solutions used for measurement of K channel gating currents were designed to minimize contamination by ionic and Na channel gating currents following the methods described by Gilly and Armstrong (1980) and White and Bezanilla (1985). Our external solution (Tris-NO₃ ASW) for K channel gating current measurements consisted of (mM) 415 TrisNO₃, 50 Ca(NO₃)₂, 10 CsNO₃, 0.2 dibucaine, 1 μM TTX, pH 7 (with 25 Tris base), and 60 sucrose, 960–980 mosmol/kg. The internal solution (Cs SIS) was (mM) 270 Cs-glutamate, 50 CsF, 360 glycine, and 10 HEPES, pH 7.4, 970 mosmol/kg. The use of internal and external Cs⁺ slows the loss of K channel function in K⁺-free solutions (Chandler and Meves, 1970; Almers and Armstrong, 1980).

**Chemical modification.** We used several chemical reagents known to react with free amino groups including the ε-amino group of lysine and terminal α-amino groups (Means and Feeny, 1971; Bell and Bell, 1988). These amino groups are usually positively charged at physiological pH levels. The reagents used included those that (a) neutralized the charge: trinitrobenzene sulfonic acid (TNBS) and acetic anhydride; (b) preserved the positive charge of the amino group: methyl acetimidate; and (c) replaced the positive charge with a negatively charged group: 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS) and succinic anhydride. Except as noted, these reagents were applied externally.

Examples of the reactions of three of these reagents with amino groups are illustrated in Fig. 1. These compounds react with the unprotonated form of the target amino groups. Conse-
quently, axons were usually treated with these reagents in 50 K ASW at pH 9. A few experiments were done at lower pH to examine the pH dependence of the reactions. Identical results were obtained with two different high pH buffers: 10 mM CHES (2-[N-cyclohexylamino]ethane-sulfonic acid) and 20 mM Na₂B₄O₇ (which replaced 40 mM NaCl).

We used two other reagents that are structurally similar to TNBS. The dinitro analogue of TNBS, dinitrobenzene sulfonic acid (DNBS), reacts more slowly with amino groups than does TNBS (Means and Feeny, 1971). Dinitrofluorobenzene (DNFB) also reacts slowly with amino groups but, in contrast to the spontaneously reversible reaction of TNBS with sulphydryl residues, reacts with sulphydryl groups to produce a stable S-dinitrophenyl derivative (Means and Feeny, 1971).

**Data analysis.** The quantitative analysis of our data included the fitting of exponential time functions to ionic and gating current records and determinations of the voltage dependence of gating charge movements and the fraction of open channels. As shown in White and Bezanilla (1985), the final approach to steady-state K channel current can be fit by a single exponential time function. Therefore, as described previously (Spires and Begenisich, 1989), we obtained an estimate of the K channel ionic current activation time constant by fitting an exponential function between 50–65% and 90–95% of the current maximum. Ionic tail currents were also fit with a single exponential time function from 90–95% of maximum to ~ 20% or less. The time constant obtained in this way is called the deactivation time constant.

The major part of K channel gating current ON and OFF responses can also be described by a single exponential time function (White and Bezanilla, 1985; Spires and Begenisich, 1989) and we fit such a function to our data. Approximately 1–2 ms of data were used for this fitting procedure. The first 20–100 μs of data were omitted from the fits to reduce contamination from the much faster, residual Na channel gating current. Since gating currents are often superimposed on residual "leakage" current pedestals (e.g., see Armstrong and Bezanilla, 1974; White and Bezanilla, 1985; Spires and Begenisich, 1989), the exponential function included a nonzero steady-state component. Specific examples of this procedure can be found in Fig. 2 of Spires and Begenisich (1989).

We estimated the voltage dependence of the fraction of open channels by a method similar to that described in Noble and Tsien (1969) but modified to correct for K⁺ accumulation in the periaxonal space (for details, see Spires and Begenisich, 1989). The voltage dependence of this normalized estimate of the fraction of open channels was fit by a two-state Boltzmann function of the form:

$$f_{\text{open}} = \frac{1.0}{1.0 + \exp \left[-q(V_m - V_{1/2})F/RT\right]}$$  \hspace{1cm} (1)$$

where $q$ represents the equivalent charge difference between the two states and $V_{1/2}$ is the voltage of the midpoint of this function. Eq. 1 was also fit to the voltage dependence of gating charge. All fits of theoretical functions used the "simplex" algorithm (Caceci and Cacheris, 1984).

**RESULTS**

**Actions of External TNBS on K Channel Ionic Currents**

The qualitative effects of external TNBS on K channel ionic currents are illustrated in Fig. 2. The currents in the left panel were obtained with 4-ms pulses to several
membrane potentials (−23, −3, 17, 37, and 57 mV). This pulse duration was long enough for the currents to reach steady levels for potentials more positive than −3 mV. The decline in current at the largest depolarizations is due to accumulation of K⁺ in the periaxonal space (Frankenhaeuser and Hodgkin, 1956). After a 6-min treatment with 2.5 mM TNBS at pH 9, the ionic currents were substantially slowed and an 8-ms pulse (right) was necessary to allow the K currents to approach steady levels.

As described in Methods, all measurements of ionic currents were done with 50 K (pH 7.4) ASW, not the elevated pH solutions used when treating with the amino group reagents. We could not detect any reversal of the TNBS-induced modifications during periods of at least an hour after return to the pH 7.4 solution. Control experiments showed that pH 9 treatment had no irreversible effects on the currents.

A quantitative analysis of the effects of TNBS treatment on the ionic current kinetics is presented in Fig. 3. In this figure the ionic current time constants before (▲) and after (■) TNBS treatment are shown at several membrane voltages. Time constants at potentials more positive than −40 mV are from ionic currents in response to depolarizations to the potentials of the abscissa. These "activation" time
constants are related to channel opening kinetics. Time constants at potentials more negative than −40 mV are from tail currents elicited by a conditioning voltage pulse of +17 mV followed by repolarization to the indicated potentials. These “deactivation” time constants are related to channel closing.

As shown in Fig. 3, treatment with TNBS slowed both activation and deactivation time constants. However, activation time constants appear to have been slowed much more than the deactivation time constants. To assess the relative effect of TNBS on activation and deactivation time constants, we computed the ratio of the values after TNBS treatment to the control values. The ratios for this and several other experiments (identified by different symbols) are illustrated in Fig. 4.

As in Fig. 3, the data at potentials more negative than −40 mV represent deactivation time constants; the data at more positive potentials are activation time constants. This figure shows that treatment with 2–2.5 mM TNBS for 5–8 min at pH 8.8–9 (open symbols) produced a maximum 2.5- to 5-fold increase in the activation time constants. In contrast, treatment with 2.5 mM TNBS at pH 7.4 for 16 min (■) had very little effect, as expected if the reaction is with the neutral (unprotonated) form of the target amino group. The kinetic changes produced by TNBS are small after only 3.5 min at pH 9 (▲) and appear to be relatively complete by 5–8 min at this pH (▲, +).

This figure also shows that TNBS produced a much larger modification of activation than deactivation time constants. Furthermore, the modification of activation kinetics was, in most cases, voltage dependent. The slowing of activation kinetics near −30 mV was relatively small and about the same as for deactivation kinetics, but increased with depolarization in a graded manner. The magnitude of the slowing then declined at the most positive potentials.
Another action of TNBS treatment can be seen in Fig. 2: the currents at the most positive potentials (and the resulting tail currents at the holding potential) were increased. One possible mechanism for this action is an effect of TNBS on the slow inactivation process of the squid axon K channels (Ehrenstein and Gilbert, 1966). This seems unlikely, since at holding potentials near −63 mV (the most positive values used), only ~15% of the K channels are inactivated (Chabala, 1984). The increase in current produced by TNBS modification was as large as 50–60% (Table I), including experiments in which the holding potential was near −70 mV where only ~5% of the channels are inactivated (Chabala, 1984). However, a more direct test was made using two different holding potentials on the same axon. At a holding potential of −78 mV there are essentially no inactivated channels, but at −48 mV ~50% of the channels are inactivated (Chabala, 1984). At a holding potential of −78 mV, TNBS treatment increased the steady-state current (determined at +62 mV) by a factor of 1.53, not very different from the 1.65-fold increase induced at a −48-mV holding potential (Table I).

**Table 1**

| Axon  | Holding potential | τ rel | \( V_{\text{m}} \), shift | q rel | I rel |
|-------|------------------|------|----------------|-------|------|
| SQDAW | −68              | 3.2  | 2.2             | 1.06  | 1.22 |
| SQDAY | −63              | 5.1  | 8.6             | 0.96  | 1.22 |
| SQDAZ | −63              | 5.2  | 7.8             | 1.05  | 1.20 |
| SQDDP | −78              | 2.7  | 8.1             | 0.93  | 1.53 |
| SQDDP | −48              | 2.9  | 7.1             | 0.98  | 1.65 |

\( \tau \) rel is the maximum value of the ratio of the activation time constant after TNBS treatment relative to control value. The shift of the midpoint of the activation curve (\( V_{\text{m}} \)) was determined by fitting Eq. 1 to the \( f_{\text{open}} \) data before and after TNBS treatment.

q rel was also obtained in the fits of Eq. 1 and is the ratio of the value obtained after TNBS treatment to control value. I rel is the amount of steady-state current increase (measured near +60 mV) produced by TNBS treatment.

TNBS and the Voltage Dependence of Channel Opening

Another possible explanation for the current increase induced by TNBS would be a shift of the voltage dependence of channel opening to more hyperpolarized potentials. This also seems an unlikely mechanism since the increase in current magnitude occurs at very depolarized potentials where there is already a very high probability of opening. Nevertheless, we determined the actions of TNBS treatment on the fraction of open channels (\( f_{\text{open}} \)) and one such determination is illustrated in Fig. 5. Data obtained before TNBS treatment are represented by triangles and data after treatment by squares. The slight shift of the voltage dependence to more positive potentials is in the wrong direction to account for the large, observed current increase. We obtained a quantitative estimate of this effect by fitting a Boltzmann
Figure 5. Voltage dependence of channel opening and TNBS treatment. Voltage dependence of $f_{\text{open}}$ (see Methods) before (▲) and after (■) an 8-min treatment with 2 mM TNBS at pH 9. Lines are fits of Eq. 1 to the data with $V_{1/2}$ values of -7 and 0.8 mV for control and TNBS data and $q$ values of 1.9e and 2.0e.

The fits of Eq. 1 also provide a quantitative determination of the voltage sensitivity of channel opening through the effective charge parameter, $q$. A value of 1.9 electronic charges was obtained before TNBS treatment, not much different than the value of 2.0e after treatment. As shown in Table I, TNBS treatment did not produce any consistent change in this parameter.

Actions of TNBS on K Channel Gating Currents

We also investigated the modifications of K channel gating currents induced by TNBS. Fig. 6 shows K channel gating currents before (left) and after (right) a 4.5-min treatment with 2.5 mM TNBS at pH 9. In this and all similar experiments, ionic currents were obtained before and after treatment in order to be sure that the extent of treatment was comparable to that previously described (e.g., Fig. 4).

The small, fast component of current apparent in Fig. 6 (left) is residual Na channel gating current, while the larger, slower current is produced by the gating of

![Figure 6. K channel gating currents and TNBS. K channel gating currents before (Control; left) and after (TNBS; right) a 4.5-min treatment with 2.5 mM TNBS at pH 9 (at 20°C). Currents in response to depolarizations to -32, -22, -12, and -2 mV (bottom to top) from a holding potential of -62 mV. Calibration: 10 μA/cm², 0.5 ms.](image-url)
K channels (White and Bezanilla, 1985; Spires and Begenisich, 1989). The apparent loss of Na channel gating current is consistent with the large reduction of Na channel conductance and Na channel gating current produced by amino group reagents (Eaton and Brodwick, 1983; Drews and Rack, 1988).

TNBS treatment produced a marked slowing of the K channel gating current OFF response as is evident in the records of Fig. 6. Less obvious were small effects on the ON response at some voltages. A quantitative measure of these observations was made by determining the time constant associated with these processes. In Fig. 7, the ratio of the time constant after TNBS treatment to that before is shown at several different voltages. Data from each of four axons are illustrated by different symbols. Relative time constants at potentials more negative than −50 mV are from the gating current OFF responses and those at more positive potentials are from the ON response.

There is too much scatter in the OFF response data in Fig. 7 to determine if there is a dependence on membrane potential, but at −62 mV the OFF time constant was increased by 1.37 ± 0.11 (SEM, n = 4), only slightly smaller than the TNBS modification of the ionic current deactivation time constants (Fig. 4). Unlike the large (2.5–5-fold) slowing of the ionic current activation time constant apparent in Fig. 4, TNBS had a very small (10–20%), possibly voltage-dependent, effect on the gating current ON response.

The records in Fig. 6 suggest that TNBS may have induced a rising phase in the gating current OFF response. However, a rising phase is also apparent in the control data of this figure, so TNBS may have exaggerated this preexisting condition. We have not systematically examined this feature of the OFF response, but White and Bezanilla (1985) have described a rising phase in the K channel gating current ON response.

TNBS treatment often increased the nonlinearity of the axon leakage current,
apparent as the nonzero current levels at the end of the pulses in Fig. 6. This increase does not compromise the estimates of gating current time constants and charge movements since the exponential fitting procedure (see Methods) allows for a nonzero, steady-state current.

**TNBS and Charge Movement**

Another effect of TNBS treatment was an apparent loss of K channel ON gating charge. The ON and OFF charge movements ($Q_{ON}$ and $Q_{OFF}$) were determined (see Methods) for three axons and the results are illustrated in Fig. 8. The ratios of $Q_{ON}$ and $Q_{OFF}$ at several potentials are shown before (triangles) and after (squares) TNBS treatment. Under normal conditions, $Q_{ON}$ and $Q_{OFF}$ are approximately equal (Spires and Begenisich, 1989) and in the figure the control data are near 1. After TNBS modification, however, $Q_{ON}$ was smaller than $Q_{OFF}$, especially at the most positive potentials.

The decrease in $Q_{ON}/Q_{OFF}$ is not likely to be due to an TNBS-induced increase in $Q_{OFF}$ since (as described below) TNBS treatment produced a net loss in the maximum value of $Q_{OFF}$. This loss of $Q_{ON}$ is probably an artifact of the exponential fitting method used to determine charge movement and is described in detail in the Discussion section.

**Multiple Components of Gating Current**

The apparent loss of ON charge suggests that there may have been two components of gating current: one that was only a little altered by TNBS treatment and a second that was slowed so much that it did not contribute to the single exponential fit. Fits of a two-exponential function to the data were unable to resolve more than one component, a common problem with multi-exponential data (e.g., see Isenberg et al., 1973). However, the data shown in Fig. 9 are consistent with the suggestion of two components of gating current.
The inset of Fig. 9 shows the voltage pulse protocol used in this type of experiment. The gating current OFF time constant is determined at the holding potential (−62 mV) after a conditioning potential (P2) to the values on the abscissa. Before TNBS treatment (triangles) the time constant from this single exponential fit was constant, independent of the P2 voltage. However, after TNBS treatment (squares), the time constant from this fit increased as P2 was made more depolarized. The ratio of the time constants after treatment to control values increased from a value of 1.5 for a P2 voltage of −32 to 1.9 at P2 = +8 mV. It may be that the component of gating current more sensitive to TNBS treatment activates at more depolarized voltages. The movement of this charge back to its resting level might produce the apparent slowing of the OFF current time constant determined by the single exponential fitting procedure.

**Figure 9.** Conditioning potential and gating current OFF time constant. Gating current OFF time constant was measured at −62 mV after a 2.5-ms conditioning pulse to several potentials. Data were obtained before (▲) and after (■) a 4.5-min treatment with 2.5 mM TNBS at pH 9. Inset illustrates the pulse protocol.

**TNBS and the Voltage Dependence of Charge Movement**

The voltage dependence of gating charge movement before (filled symbols) and after (open symbols) TNBS treatment is illustrated in Fig. 10. For each of these three experiments (represented by different symbol types), the charge–voltage relations were determined using only \( Q_{OFF} \) to avoid the apparent loss of \( Q_{ON} \) (see text above and Fig. 8). A Boltzmann function (Eq. 1 with \( Q_{max} \) rather than 1.0 as the ordinate maximum) was fit to these data. The \( Q_{max} \) values so obtained were used to normalize the charge movements. For these three experiments, the \( Q_{max} \) values after TNBS were reduced by 19, 30, and 50%. These values were probably not different than the reduction expected without TNBS treatment due to the length of the experiments and the many solution changes (for measurement of ionic and gating currents before and after TNBS treatment and for the treatment itself).

There does not appear to be any systematic change in the voltage dependence of
charge movement produced by TNBS treatment (Fig. 10). Neither the steepness of
this relation nor its position on the voltage axis appear to be altered by TNBS

treatment. The solid line in the figure is a fit of the Boltzmann relation (equivalent to
Eq. 1) to all the data. The midpoint, $V_{1/2}$, and steepness, $q$, of this fit were $-29$ mV
and $2.0e$, respectively, not much different than the $-38$ mV and $2.2e$ values that were
previously obtained in a much more extensive study (Spires and Begenisich, 1989).

**Specificity of Modification**

In addition to reacting readily with amino groups, TNBS also modifies sulfhydryl
residues, but the S-trinitrophenyl derivative is unstable and the original sulfhydryl
residue is spontaneously restored (Freedman and Radda, 1968; Means and Feeny,
1971). The apparent irreversibility of the TNBS effects reported here are consistent
with amino but not sulfhydryl modification. The pH dependence of the TNBS effects

(Fig. 4) is also consistent with amino group modification. To provide more informa-

tion on chemical specificity, we determined the effects of several other reagents on K
channel properties. A summary of these findings is presented in Table II.

The first column of Table II lists five of the reagents we used that are known to
react with amino groups (Means and Feeny, 1971; Bell and Bell, 1988). These are
arranged according to the resultant modification (see Methods and Fig. 1) of the
amino group that is normally positively charged at physiological pH. Also shown are
other reactions commonly found for these reagents. TNBS produced the largest
effects on K channels, but some other compounds, especially methyl acetimidate, also
produced substantial effects. The data in this table add further support to the
suggestion that the TNBS-induced effects are due to modification of amino groups.
To further test that the TNBS actions were due to amino group modification, we
treated axons with two other reagents that are structurally similar to TNBS (see
DNBS reacts much more slowly with amino groups than TNBS and was without effect on K channels. DNFB also reacts slowly with amino groups but readily reacts with sulphydryl groups to produce a stable S-dinitrophenyl derivative and was also without effect on K channel properties. These results are consistent with a specific modification of amino groups by TNBS and not, for example, modification of sulphydryl residues.

The Number of Amino Groups Modified

In an effort to determine the number of kinetically distinguishable amino groups modified by TNBS treatment, we measured the time course of the slowing of the ionic current activation time constant and the increase in steady-state current. The relative increase of these two parameters is shown in Fig. 11 as a function of time during treatment. Since the TNBS modifications were very slow at pH values near 7 (Fig. 4), the data illustrated in the figure were obtained entirely at pH 9. At time zero, 2 mM TNBS was added to the bath solution. After the short delay necessary for chamber solution change, the steady-state current started to increase (filled squares). The increase in current appears to occur in two phases and seems to reach a constant value by the end of the measurement period. The activation time constant (open squares) increased more slowly and did not reach a constant value by the end of the measurement period.

The potassium channel current during this TNBS treatment will necessarily be from a heterogeneous population of modified and unmodified channels. Consequently, given the difficulties of separating exponential components from such records (as described above), it is not possible to identify the specific number of modified amino groups on each channel. Furthermore, the TNBS-induced alterations in channel conformational change molecular rate constants may be reflected in current amplitude and time constant values in a complex way. Nevertheless, a simple interpretation of the multi-phase time course of the increase in current amplitude and the generally slower increase in the current time course is the presence of several

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**Table II**

| Reagent       | N  | Charge | −NH₂ | −SH | Histidyl | I       | τ       | ΔV₁/₂ |
|---------------|----|--------|------|-----|----------|--------|--------|-------|
| TNBS          | 15 | 0      | +++  | R   | −        | ++     | +++    | ++    |
| Acetic anhydride | 5  | 0      | +++  | R   | R        | −      | +      | +     |
| Methyl acetimidate | 7  | +      | +++  | −   | −        | +      | ++     | ++    |
| SITS          | 2  | +      | +++  | −   | −        | +      | +      | +     |
| Succinic anhydride | 4  | −      | +++  | R   | −        | −      | −      | −     |

N is the number of experiments. Charge is the effective charge of the modified amino group. The relative reactivity of the reagents with amino (−NH₂), sulphydryl (−SH), and histidyl groups are shown. R represents spontaneously reversible reactions. I represents the relative increase in steady-state ionic current. τ represents the relative slowing of the ionic current activation time constant. ΔV₁/₂ represents the relative shift of the midpoint of the activation (fₐ₉¹) curve. Except in the charge column, − represents no effect and the relative positive effects are represented by +.
different amino groups on squid axon K channels. In this view these amino groups are involved in different aspects of the channel conformational change process and react with TNBS with distinguishable rates.

DISCUSSION

Summary of Results

The results presented here show that several reagents known to react with amino groups produced modifications in several properties of the delayed rectifier K channel in squid giant axons. These modifications included an increase in steady-state current amplitude, a shift of the activation curve toward more positive potentials, and a slowing of both activation and deactivation (tail) kinetics. K channel gating currents were also affected, including an apparent reduction in the $Q_{on}/Q_{off}$ ratio. The voltage sensitivity of activation and of channel charge movement, as judged by the effective charge term of Eq. 1 (see Figs. 5 and 10 and Table I), were not altered.

Several observations (the pH dependence in Fig. 1; Table II and the associated text) provide evidence that amino groups were the targets of TNBS modification. We have previously reported that histidine modification also slows the time constant for K channel activation (but not deactivation) and shifts the activation curve toward positive potentials (Spires and Begenisich, 1990). The high specificity of methyl acetimidate suggests that the results reported here are due to modification of amino and not histidyl residues. Furthermore, we found that modification of K channel kinetics with the histidine reagent, diethyl pyrocarbonate, occurred even after TNBS modification (data not shown).

There appears to be more than one amino group involved in the modifications
reported here (see Fig. 11). These groups are probably located on the external surface of the K channel since TNBS crosses cell membranes only extremely slowly (Gordesky et al., 1975; Haest and Deuticke, 1975). Internal application of TNBS produced a large reduction in K channel currents with little or no effect on kinetics (data not shown).

Comparison with Earlier Results

There have been several recent studies using group-specific reagents to determine what amino acids play important roles in the voltage dependent gating of ionic channels (Oxford et al., 1978; Cahalan and Pappone, 1981, 1983; Eaton and Brodwick, 1983). Some studies have included measurement of gating as well as ionic currents, though these have focused exclusively on Na channels (Meves and Rubly, 1987; Drews and Rack, 1988; Meves et al., 1988). Cahalan and Pappone (1983) showed that TNBS produces very large kinetic modifications of K channels in frog myelinated nerve. Both activation and deactivation time constants were increased by TNBS treatment, but deactivation was slowed substantially more than activation—just the opposite of what we found for squid nerve K channels. The amino acid sequence for neither the squid axon nor the frog myelinated K channel has been determined. Consequently, we cannot relate the differences in TNBS modification to specific structural differences in these two channels.

Mechanism of Amino Group Modification

The main effects of amino group modification were: (a) an increase in the size of ionic currents at large positive voltages; (b) a slowing of the kinetics of macroscopic ionic currents, especially the activation time constant; (c) a shift of the voltage-dependent probability of channel opening to more positive potentials but without effect on the voltage sensitivity; and (d) a differential slowing of one component of K channel gating current producing an apparent loss of $Q_{ON}$ at more depolarized potentials.

The observed increase in current was shown not to involve the slow inactivation process. The increase could be produced by an increase in the number of channels, an increase in the probability of channel opening, and/or an increase in the single channel current. While an increase in the number of channels produced by amino group modifying reagents seems unlikely, a complete understanding of this issue awaits measurement of TNBS modification of axon single K channel currents.

The results of amino group modification did not depend on a change in the charge of the modified amino group (Table II), but of the two reagents that produce a negatively charged modification only SITS produced any alterations in K channel properties, and these were small. Since there were clear effects on some properties of gating currents by amino group neutralization with TNBS, it might be concluded that amino groups must be part of the channel voltage sensor (see also below). However, these effects could also occur if there were coupling between voltage-sensitive and -insensitive conformational changes. Furthermore, there was little or no effect on the voltage sensitivity of channel opening or of gating charge moved. Taken together, these observations suggest that the modified amino groups probably contribute relatively little, if at all, to the overall gating charge.
A K Channel Kinetic Model

We have previously described a kinetic model for K channels which was developed to account for the measured properties of both ionic and gating currents (Spires and Begenisich, 1989). Many of the results of amino group modification on ionic and gating currents can be understood in the context of this model.

In the model, the K channel is composed of two independent, heterologous subunits, each of which can exist in two conformations:

\[ 1 \leftrightarrow 2 \quad A \leftrightarrow B \]

In this model a channel can conduct only if the subunits are in the 2 and B states. For a population of such channels the fraction of open channels is given by:

\[ f_{\text{open}} = P_2 \cdot P_B \]

where \( P_2 \) and \( P_B \) are the probabilities of the subunits being in states 2 and B, respectively. The time dependence of these probabilities has the simple form:

\[ P_2(t) = P_2^0 - (P_2^0 - P_2^0) \exp \left(-t/\tau_{12}\right) \]

\[ P_B(t) = P_B^0 - (P_B^0 - P_B^0) \exp \left(-t/\tau_{AB}\right) \]

where \( P_2^0 \) and \( P_B^0 \) denote the steady-state probabilities, \( P_2^0 \) and \( P_B^0 \) represent the initial values, and \( \tau_{12} \) and \( \tau_{AB} \) are the voltage-dependent time constants for the 1 \( \leftrightarrow \) 2 and A \( \leftrightarrow \) B transitions, respectively.

The steady-state probabilities have the following form:

\[ P_2^0 = 1/(1 + \exp \left[-q_{12}(V_m - V_{12})F/RT]\right]) \]

\[ P_B^0 = 1/(1 + \exp \left[-q_{AB}(V_m - V_{AB})F/RT]\right]) \]

where \( q_{12} \) and \( q_{AB} \) are the amount of charge moved in the 1 \( \leftrightarrow \) 2 and A \( \leftrightarrow \) B transitions, respectively. \( V_m \) is the membrane potential and \( V_{12} \) and \( V_{AB} \) are the midpoints of the charge distributions.

The gating current is the sum of the gating currents from each of the two subunits:

\[ I_g = I_{12}^g + I_{AB}^g \]

\[ I_{12}^g = q_{12}/\tau_{12} \cdot (P_2^0 - P_2^0) \exp \left(-t/\tau_{12}\right) \]

\[ I_{AB}^g = q_{AB}/\tau_{AB} \cdot (P_B^0 - P_B^0) \exp \left(-t/\tau_{AB}\right) \]

The 1 \( \leftrightarrow \) 2 transition was assigned a charge movement, \( q_{12} \), of 3e with a midpoint, \( V_{12} \), of \(-45 \text{ mV}\), and given time constant values, \( \tau_{12} \), equal to the measured gating current time constants. The A \( \leftrightarrow \) B subunit was given values of 1.5e and \(-5 \text{ mV}\) with the time constants of the K channel ionic current kinetics. With these properties for the two subunits, the model qualitatively accounted for the large voltage displacement between steady-state charge movement and channel activation (White and Bezanilla, 1985; Spires and Begensich, 1989) and for the observed difference in ionic current and gating current time constants at membrane potentials between approximately \(-30 \text{ and } 0 \text{ mV}\) (Spires and Begensich, 1989).

Eqs. 3–5 demonstrate that the amplitude of each gating current component
depends on the voltage range over which the charge moves, and varies directly with the amount of charge moved but inversely with the time constant. In terms of this model, ON gating currents at potentials near (and more negative than) $-30 \, \text{mV}$ will almost entirely reflect the $1 \leftrightarrow 2$ transition because: (a) this transition has two-thirds of the total charge; (b) the time constant is equal to or faster than the $A \leftrightarrow B$ transition; and (c) the $A \leftrightarrow B$ transition activates at much more positive potentials.

At positive potentials, each ON component will have approximately the same time course because the ionic and gating current time constants are equal (White and Bezanilla, 1985; Spires and Begenisich, 1989). At these potentials the amplitude of the $1 \leftrightarrow 2$ component will represent approximately two-thirds of the total since it has two-thirds of the total charge.

For OFF responses measured near $-60 \, \text{mV}$, the two components will also have similar time courses due, again, to the similarity in ionic and gating current kinetics. The relative portion of each component will depend on the activation potential (and the time at that potential), but the larger amount of the charge in the $1 \leftrightarrow 2$ transition assures its larger contribution.

Most of the actions of TNBS modification can be accounted for by this model. The results of Fig. 4 can be reproduced by the model with a three- to fourfold increase of $\tau_{AB}$ at potentials more positive than $-40 \, \text{mV}$ and a much smaller (1.5-fold) increase at negative potentials. Alternatively, there could be differential effects on the forward and reverse rate constants, but such considerations are beyond the scope of this simple model (see below).

Since the $A \leftrightarrow B$ subunit contributes relatively little gating current (at most one-third of the total), the TNBS modifications of gating currents are predicted to be more subtle than the three- to fourfold effects on ionic current. Our findings were consistent with this prediction.

The small slowing of the measured gating current OFF kinetics apparent in Fig. 7 can be accounted for by a small increase of $\tau_{12}$ at potentials more negative than $-50 \, \text{mV}$ (or by differential actions of TNBS on the forward and reverse rate constants of the $1 \leftrightarrow 2$ transition). But perhaps the more intriguing aspect of TNBS modification is the apparent loss of ON charge movement at positive potentials (Fig. 8). The model is also consistent with this observation.

As previously noted, essentially all the gating charge measured at potentials more negative than $-30 \, \text{mV}$ arises from the $1 \leftrightarrow 2$ subunit. Consequently, $Q_{ON}$ will equal $Q_{OFF}$ at these voltages, even after TNBS modification. However, at positive potentials, the large TNBS-induced increase in $\tau_{AB}$ will make the ON gating current component from this subunit so small it will not contribute to the single exponential fits and so the actual charge movement will be artifactually underestimated. This computational artifact will not occur when determining $Q_{OFF}$ due to the similar values of $\tau_{AB}$ and $\tau_{12}$ at potentials near and more negative than $-60 \, \text{mV}$. A limiting value of the (incorrectly) computed $Q_{ON}/Q_{OFF}$ can be estimated from the model: it will simply be the $q_{10}/(q_{10} + q_{AB})$ or 0.67, not far from the value of $\sim 0.6$ seen in Fig. 8.

In addition to accounting for many of the normal properties of ionic and gating currents (Spires and Begenisich, 1989) and most of the actions of TNBS described here, this simple model was successful in reproducing the observed effects of histidine group modification of $K$ channels (Spires and Begenisich, 1990). However,
there are several important limitations of the model in its present form which have been previously described (Spīres and Begenisich, 1989, 1990). As noted above, the model lacks a detailed description of the forward and reverse rate constants governing the subunit transitions. The model in its present form cannot account for the existence of a gating current rising phase, but, as discussed in Spīres and Begenisich (1989), could produce a rising phase if each subunit consisted of three states.

A more significant objection to the model is that rather than two heterogeneous subunits, some K channels can be formed of four homogeneous subunits (MacKinnon, 1991). It is not known whether or not native channels are formed of homologous subunits, but heteromultimeric channels can be produced by in vitro expression systems (Isacoff et al., 1990; Ruppersburg et al., 1990). Furthermore, it appears that some types of brain K channels are formed from peptides coded by more than a single species of mRNA (Rudy et al., 1988).

A more complete model than the one described here will need to address all these issues and still be able to account for the many differences observed between ionic current and gating current behavior. To date, it seems clear that the simple model with heterologous, independent subunits has been more successful than more complex models that consider a tight coupling of channel conformational states (White and Bezanilla, 1985; see Zagotta and Aldrich, 1990, for a detailed discussion of these issues).

Functionally Important Amino Groups

The lack of amino acid sequence information for the squid K channel makes it impossible to relate the observed effects of amino group modification to specific locations on this molecule. However, since the reagents we used do not react with arginine amino groups, the observed modifications were probably of the ε-amino group of lysine and/or the protein terminal α-amino group. The groups modified in this study are certainly on the external surface of the channel protein and easily accessible to TNBS and some of the other reagents. It may be useful to identify lysine residues on the (proposed) external surface of K channels for which sequence data are available.

Several homologous cDNA species have been isolated from rat cortex (Stühmer et al., 1989). These cDNAs code for potassium channels (RCK) with very different kinetic properties. The variable (nonconserved) sections of the predicted amino acid sequences are in the extracellular (Pongs et al., 1988) regions linking the proposed transmembrane segments S1 and S2, S3 and S4, and S5 and S6. There are only two lysine residues in these regions: between S3 and S4 of RCK1 and between S5 and S6 of RCK4. Given the large kinetic effects we observed with amino group reagents, these lysine residues may be interesting candidates for site directed mutagenesis.

The lysine (and arginine) groups in the S4 segment of voltage-gated channels may play an important role in the gating of these channels. Papazian et al. (1991) made mutations in the S4 region of Shaker B (inactivating) K channels. These mutations included substituting the neutral amino acid glutamine for each of the two lysine residues in this segment. One of these mutations (Lys 374) did not produce functional channels in the Xenopus oocyte expression system. While neutralization of
the other lysine (Lys 380) has no effect on the voltage dependence of steady-state activation and inactivation, there is a slight increase in the rate of channel activation (and inactivation), a change opposite to the effects of TNBS. This may represent a difference in K channel types or may be because TNBS acts on amino groups outside the S4 segment. To address this issue, we have used the baculovirus expression system to study the actions of TNBS on channels very similar in sequence to the Shaker B clone (Klaiber et al., 1990). We found that TNBS treatment of these channels (Spries, S., and T. Begenisich, unpublished observations) slowed activation (and inactivation), similar to its effects on squid axon delayed rectifier K channels. So it seems that there may be lysine residues not in the S4 segment that play an important functional role in several types of K channels.

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