

Modification of Potassium Channel Kinetics by Histidine-specific Reagents

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ABSTRACT We have examined the actions of histidine-specific reagents on potassium channels in squid giant axons. External application of 20–500 μM diethylpyrocarbonate (DEP) slowed the opening of potassium channels with little or no effect on closing rates. Sodium channels were not affected by these low external concentrations of DEP. Internal application of up to 2 mM DEP had no effect on potassium channel kinetics. Steady-state potassium channel currents were reduced in an apparently voltage-dependent manner by external treatment with this reagent. The shape of the instantaneous current–voltage relation was not altered. The voltage-dependent probability of channel opening was shifted toward more positive membrane potentials, thus accounting for the apparent voltage-dependent reduction of steady-state current. Histidine-specific photo-oxidation catalyzed by rose bengal produced alterations in potassium channel properties similar to those observed with DEP. The rate of action of DEP was consistent with a single kinetic class of histidine residues. In contrast to the effects on ionic currents, potassium channel gating currents were not modified by treatment with DEP. These results suggest the existence of a histidyl group (or groups) on the external surface of potassium channels important for a weakly voltage-dependent conformational transition. These effects can be reproduced by a simple kinetic model of potassium channels.

INTRODUCTION

Potassium channels serve a variety of roles in excitable tissues. Current through K channels in axons helps to terminate the action potential. Rapidly inactivating K channels regulate repetitive action potential activity in neurons (e.g., Conner and Stevens, 1978). Ca^{2+} -activated K channels control bursting behavior in many cell types (e.g., Meech and Standen, 1975). Transmitter-modulated K channels may underlie simple forms of learning (e.g., Alkon, 1979; Siegelbaum et al., 1982).

The amino acid sequences of several putative K channels have been determined (Kamb et al., 1987; Tempel et al., 1987; Schwarz et al., 1988; Baumann et al., 1988; Tempel et al., 1988; Butler et al., 1989). Some of these channels have been functionally expressed in *Xenopus* oocytes (Stuhmer et al., 1988; Timpe et al., 1988; Christie et al., 1989). In spite of this work, functionally important chemical groups

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have not yet been identified. Consequently, information on this aspect of K channels has come from somewhat indirect methods. For example, from the effects of external pH on K channel kinetics, Shrager (1974) suggested the involvement of a histidine residue in channel gating. He also noted that the histidine-modifying reagent diethylpyrocarbonate (DEP) slowed and reduced K channel currents with little or no effect on Na channel currents.

We have further investigated the actions of DEP on K channel kinetics. We have quantitatively examined the effects of this reagent on both the activation and deactivation kinetics, on instantaneous and steady-state ionic currents, and on K channel gating currents. We have also studied the effects of another compound (rose bengal) known to modify histidine groups.

We found that these two reagents slow the activation of K channels with little or no effect on deactivation time course. Treatment with these compounds did not alter the shape of the instantaneous current-voltage relation. DEP also had no measurable effect on K channel gating currents. These results suggest the existence of a histidyl group (or groups) on the external surface of potassium channels important for a weakly voltage-dependent conformational transition.

A preliminary report of these findings was presented at the annual meeting of the Biophysical Society (Spires and Begenisich, 1985).

METHODS

Biological Preparation

The data in this report were obtained with giant axons from the squid *Loligo pealei* available 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 Begenisich, 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 room temperature (20–22°C). Series resistance compensation was used.

Membrane currents were measured with a 12 bit analogue/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/analogue 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 subtraction potential for the P/4 pulses was the holding potential; a subtraction 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 was a high potassium artificial sea water (50 K ASW) containing (in mM): 390 NaCl, 50 KCl, 10 CaCl₂, 50 MgCl₂, 10 HEPES buffer, with pH

near 7.4. The elevated potassium was used to reduce the effects of K^+ ion accumulation in the periaxonal space surrounding these axons (Frankenhaeuser and Hodgkin, 1956). Na^+ channel currents were blocked by addition of 1 μM tetrodotoxin (TTX). In some experiments a pH 6 solution was used that was identical to the 50 K ASW but 10–12 mM MES replaced HEPES. The external solutions had osmolarities of ~ 975 mosmol/kg. The standard internal solution (K SIS) contained (in mM): 50 KF, 270 K glutamate, 15 K_2HPO_4 , 390 glycine, with pH of 7.4 and 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 conditions described by Gilly and Armstrong (1980) and White and Bezanilla (1985). Gilly and Armstrong (1980) showed that Na channel currents (but not K channel currents) are inhibited by low (0.2–0.5 mM) concentrations of the local anesthetic dibucaine. White and Bezanilla (1985) found that replacing external Cl^- by NO_3^- selectively reduces Na channel ionic and gating currents. These authors used a K^+ -free internal solution containing *N*-methylglucamine as the major cation. Gilly and Armstrong (1980) found that a component of gating current is lost when axons are perfused with K^+ -free media, consistent with the earlier work of Chandler and Meves (1970) and Almers and Armstrong (1980). The destruction of K channels by K^+ -free solutions is greatly slowed by internal and external Cs^+ ions (Chandler and Meves, 1970; Almers and Armstrong, 1980).

Because of these considerations, we have used somewhat modified internal and external solutions (see Spires and Begenisich, 1989) for measurements of K channel gating currents. Our external solution (Tris- NO_3 ASW) for K channel gating current measurements consisted of (in mM): 415 Tris- NO_3 , 50 $Ca(NO_3)_2$, 10 $CsNO_3$, 0.2 dibucaine, 1 μM TTX, pH 7 (with 25 Tris base), 60 sucrose, 960–980 mosmol/kg. In several experiments gating currents were measured at pH 6; in these experiments the Tris- NO_3 ASW was modified to include 10 or 20 mM MES. The internal solution (Cs SIS) was (in mM): 270 Cs-glutamate, 50 CsF, 360 glycine, 10 HEPES, pH 7.4, 970 mosmol/kg.

Chemical modification. DEP reacts with histidyl residues in many model peptides and native proteins to yield an *N*-carbethoxyhistidyl derivative (see reviews by Miles, 1977; Lundblad and Noyes, 1984). At high reagent excess, a disubstituted derivative is formed. Even though the reaction is with the neutral form of the imidazole of histidine, it proceeds readily and with high specificity at pH 6 and the *N*-carbethoxyhistidyl residues are more stable at acid pH. DEP is subject to hydrolysis in aqueous solutions with a half-time that depends on temperature, pH, and the type of pH buffer used. At 25°C in a Na phosphate-buffered solution the half-time is 24 min at pH 6 and 9 min at pH 7 (Berger, 1975). Ehrenberg et al. (1976) reported a half-time of ~ 28 min in a neutral aqueous solution at 25°C and ~ 61 min at 15°C. In our experiments DEP (0.1–0.5 mM) was added to a pH 6 solution (described above) just before application to the axon at 15°C (ionic current experiments) or 20–22°C (gating currents). To minimize differences between control and treatment conditions, we usually used a pH 6 solution before and after as well as during treatment with DEP. In some experiments data were obtained at pH 7.4 before and after treatment with DEP applied at pH 6. In all cases, DEP was applied in a constant flowing solution.

Photo-oxidation catalyzed by the dye rose bengal has been used to specifically modify histidyl residues (Lundblad and Noyes, 1984). We used 10–100 μM rose bengal at pH 7.4; most experiments were with 20 or 25 μM . The light source for irradiation was a quartz halogen lamp; exposure of the axon was via glass fiber optic light pipes.

Data analysis. The quantitative analysis of our data includes the fitting of exponential time functions to ionic and gating current records and the determination of instantaneous ionic currents, 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

accurately represented 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 current tail currents were likewise fit from 90–95% of maximum to ~20%. The major part of K channel gating current can also be described by a single exponential time function (White and Bezanilla, 1985; Spires and Begenisich, 1989).

We determined the voltage dependence of the fraction of open channels as described in Spires and Begenisich (1989). The voltage dependence of the fraction of open channels was fit by a two-state Boltzmann function of the form

$$1.0/(1.0 + \exp[-q(V_m - V_{1/2})F/RT]), \quad (1)$$

where q represents the equivalent charge difference between the two states; $V_{1/2}$ is the voltage of the midpoint of this function. Instantaneous K channel currents were obtained as the amplitude of the exponential function fit to the currents after a brief, activating depolarization. K channel gating charge movements were determined from the amplitude and time constant of the gating current records (White and Bezanilla, 1985; Spires and Begenisich, 1989). All fits of theoretical functions used the “simplex” algorithm (Caceci and Cacheris, 1984).

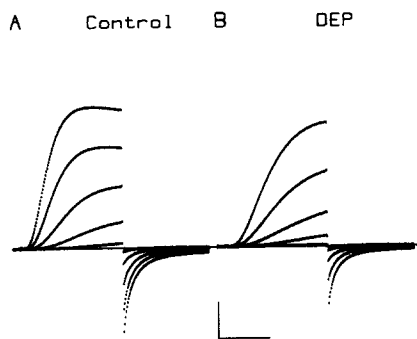


FIGURE 1. K channel ionic current modification by DEP. Currents elicited by depolarizations from a holding potential of -73 mV to -13 , 7 , 27 , 47 , and 67 mV. *A*, K channel currents in pH 6, 50 K ASW before application of DEP. *B*, Currents after a 5.5-min. treatment with $350 \mu\text{M}$ DEP in pH 6, 50 K ASW. Calibration: 2 mA/cm^2 , 2.0 ms .

RESULTS

Actions of DEP on K Channel Kinetics

The most apparent action of DEP on K channels is illustrated in Fig. 1. *A* shows K channel currents recorded in 50 K, pH 6 ASW. These currents were obtained with 4-ms pulses to several different membrane potentials (-13 , 7 , 27 , 47 , and 67 mV). The currents in *B* were obtained in the control ASW after 5.5-min treatment with $350 \mu\text{M}$ DEP. The most noticeable effect was the substantial slowing of the current activation. The actions of DEP were not reversed by washing with control solutions at pH 6 or 7.4 for up to 2 h.

The actions of DEP were entirely specific for K channels. Application of up to 5 mM for 7 min had no effect on Na channel kinetics and only a small reduction in current amplitude consistent with the results of Oxford et al. (1978). These authors reported only a 27% reduction of Na channel current by 1-mM treatment for 30

min. DEP only affected K channel currents when applied externally. Internal application of 2 mM DEP for 6.5 min at pH 6 produced only a 1–2% reduction of current amplitude with no effect on channel kinetics.

The DEP-induced modifications of K channel kinetics illustrated in Fig. 1 were too large to analyze quantitatively with the 4-ms voltage pulses used in that experiment. Data from another experiment with 12-ms pulses are presented in Fig. 2. Currents from three voltages (–3, 22, and 47 mV) before and after 100- μ M DEP treatment for 12 min are superimposed. The slowing of the activation kinetics seen in Fig. 1 is also apparent here. This slowing was examined quantitatively by fitting a single exponential function to the later part of the records (between the limits indicated by the arrows). The resultant fits (solid lines) are superimposed on the data. The time constants obtained by these fits and from those at other potentials are presented in Fig. 3.

The circles in Fig. 3 represent K channel activation time constants before (open circles) and after (solid circles) treatment with DEP. A two- to threefold slowing of

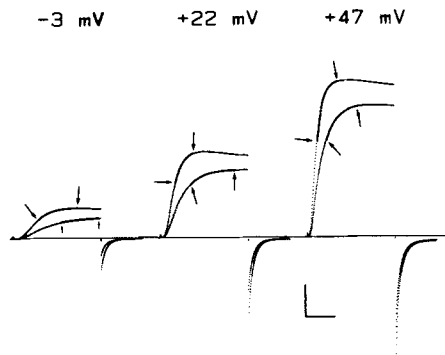


FIGURE 2. K channel ionic currents and DEP with longer test depolarizations. 12-ms depolarizations to the potentials indicated. Currents are shown before (larger) and after DEP treatment (100 μ M for 12 min). The results of exponential fits are illustrated by solid lines superimposed on the data points. The arrows indicate the parts of the records used for the exponential fitting procedure. The P/4 subtraction procedure (see Methods) was not used. Calibration: 2 mA/cm², 4 ms.

the activation time constant is apparent. The slowing effects of DEP cannot be accounted for by any reasonable shift of these data along the voltage axis.

Also shown in Figs. 1 and 2 are K channel currents recorded after the membrane voltage is stepped back to the holding potential. These tail currents reflect the channel closing process and appear less affected by DEP than the activation or opening kinetics. The kinetics of these tail currents can be analyzed by fitting single exponential functions to the data. The time constants at the holding potential of –68 mV obtained from the data of Fig. 2 are illustrated as triangles in Fig. 3. The time constants before and after DEP treatment at this potential are essentially identical.

Tail current time constants (triangles) from another experiment with a larger voltage range are shown in Fig. 4. The tail time constants after DEP treatment (solid triangles) appear the same as those obtained before treatment (open triangles). The circles in this figure show that in this same experiment there was a large effect of DEP (solid circles) on the K channel activation time constants. This figure further

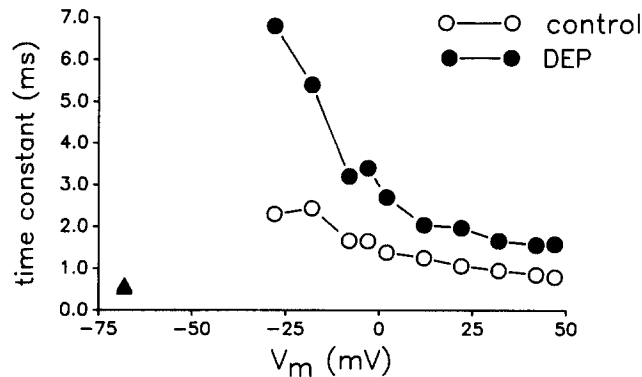


FIGURE 3. Time constants of K channel ionic currents. Time constants were derived from exponential fits as in Fig. 2. Values before (open circles) and after (solid circles) DEP treatment are shown at membrane potentials between about -25 and $+50$ mV. Time constants from fits to tail currents at -68 mV are also shown (triangles).

demonstrates that the actions of DEP are not consistent with simply a shift of membrane voltage.

DEP Treatment and K Channel Current Amplitude

DEP reduced the steady-state level of K channel ionic currents. This reduction was consistently much less at positive potentials than at more negative values. In some cases (e.g., Fig. 1) there was little or no reduction at potentials more positive than 50 mV, but in some experiments there was as much as a 15% reduction in steady-state current (e.g., Fig. 2). In those experiments in which it occurred, much of the reduction at these positive potentials was probably due to the inevitable, slow loss of ion channel currents during experiments, but a small, variable, direct effect of DEP cannot be ruled out.

The voltage-dependent reduction of steady-state K current was, however, a direct result of treatment with DEP. There are two general mechanisms by which this could occur: (a) a change in the rectification of the open channel current-voltage relation, or (b) a change in the voltage dependence of channel opening. The first mechanism

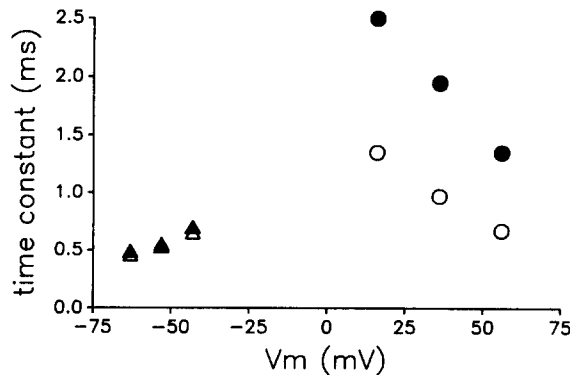


FIGURE 4. Activation and deactivation time constants of K channel ionic currents. Time constants before (open symbols) and after (solid symbols) treatment with DEP ($350 \mu\text{M}$ for 4 min) are shown. Both activation (circles) and deactivation (triangles) data are illustrated.

could be tested directly with single channel currents or indirectly by measuring instantaneous channel currents.

Actions of DEP on the Instantaneous Current–Voltage Relation

Fig. 5 shows instantaneous K channel currents obtained at several potentials after a depolarizing pulse to 17 mV. The open circles were obtained before DEP treatment. The activating voltage pulse was of a relatively small duration to minimize accumulation of K^+ in the periaxonal space. Nevertheless, the reversal potential of the current was -36 mV, somewhat depolarized from the expected value of -48 mV for the solutions used. The solid circles represent currents obtained after DEP treatment. A reduction of current is expected since the kinetic slowing produced by DEP will result in fewer channels opened by the conditioning voltage pulse. The reversal potential of the current after DEP was -38 mV consistent with less K^+ accumulation produced by the smaller current. The solid triangles in this figure are the result of

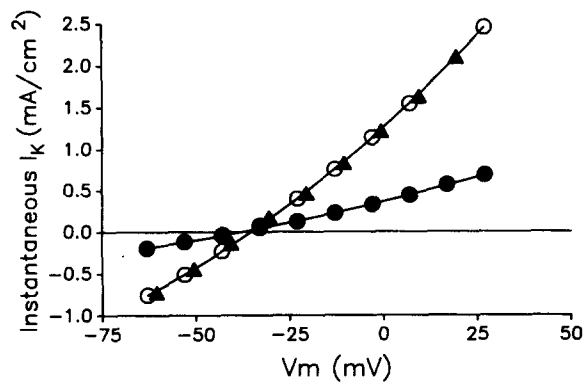


FIGURE 5. Instantaneous current–voltage relations and DEP. Instantaneous currents are illustrated before (open circles) and after (solid circles) a 4-min treatment with $350 \mu\text{M}$ DEP. A 3-ms pulse to $+17$ mV was used to open K channels. The triangles are the result of shifting the DEP data along the voltage axis by 2.5 mV and then scaling the currents by a factor of 3.7 . The solid lines are fits of second order polynomials to the data and have no theoretical significance.

shifting the DEP data by 2.5 mV (to correct for accumulation) and then scaling by a factor of 3.7 . It is evident that the apparent voltage-dependent reduction of steady-state current by DEP cannot be accounted for by a change in the rectification in the open channel current–voltage relation.

Actions of DEP on the Voltage Dependence of Channel Opening

A test of the alternate mechanism is illustrated in Fig. 6. The fraction of open channels was determined before (open circles) and after (solid circles) DEP treatment. The solid lines are the results of fits of a Boltzmann function to the data. The midpoint of this function for the control data was -3 mV. This value is slightly more positive than the -7 mV value reported in Spires and Begenisich (1989), consistent with the shift expected to be produced by the pH 6 solution (Shrager, 1974; Carbone et al., 1978). The data after DEP treatment (solid circles) are seen to be shifted to more positive potentials. The midpoint of the fitted function was $+4$ mV,

representing a 7-mV shift in the open channel voltage dependence. This effect is consistent with the DEP-induced, voltage-dependent reduction of steady-state current.

The voltage sensitivity of the $f_{\text{open}} - V_m$ relation did not appear to be altered by DEP. A measure of this is the charge parameter, q , in the Boltzmann function (Eq. 1). The control data of Fig. 6 are fit by a charge of $2e$ as are the data after DEP treatment. This value is not appreciably different than the average value of $1.6e$ that we have previously reported for pH 7.5 solutions (Spires and Begenisich, 1989).

Reagent Specificity

We also investigated the effects of another known histidine reagent on K channel activity. Fig. 7 shows that the actions of rose bengal on K channel function were the same as those produced by DEP. Panel A shows that activation time constants were

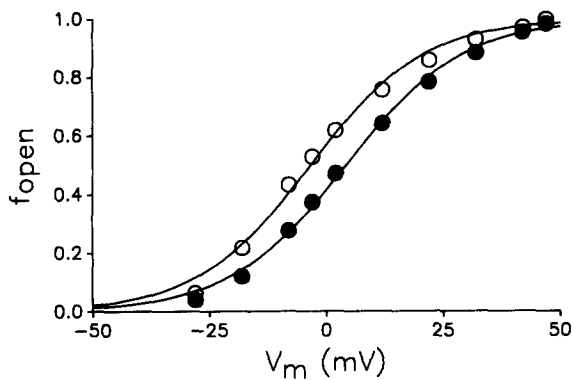


FIGURE 6. Voltage dependence of the fraction of open channels and DEP. Data before (open circles) and after (solid circles) DEP treatment ($100 \mu\text{M}$ for 12 min) are shown. Voltage pulses of 12 ms to the voltages on abscissa were used. The solid lines are the best-fit Boltzmann functions to the data. The midpoints of the functions obtained from these fits were -3 and 4 mV before and after DEP treatment, respectively. The steepness of these functions (in terms of the number of equivalent electronic charges) was $2.0e$ for both sets of data.

increased by treatment with rose bengal but tail time constants were unaffected. Panel B shows that, like DEP, rose bengal treatment produced a small shift of the voltage dependence of channel opening to positive potentials. The marked similarity between the actions of DEP and rose bengal indicates that these two reagents are most likely modifying the same chemical groups.

The Number of Chemical Groups Modified

It would be of use to directly determine the number of chemical groups modified by DEP. This requires specifically isolating K channel proteins from axons. Such isolation for K channels has not yet been reported. However, it may be possible to analyze the rate of DEP reaction to determine the number of kinetically distinguishable chemical groups. To obtain data for such an analysis we treated axons with a relatively low ($100 \mu\text{M}$) concentration of DEP for ~ 12 – 14 min. Fresh DEP was

added after 5 min to maintain a constant DEP concentration in spite of the ongoing hydrolysis (see Methods). The rate of reaction was assayed by changes in the activation time constant at three membrane potentials (-3 , 22 , and 47 mV). The time constants reached steady values after ~ 9 min. Changes in the time constants were normalized to their respective maximum values and plotted semilogarithmically in Fig. 8. The relatively linear relation of this plot suggests a single chemical reactive residue or several residues with indistinguishable kinetics. However, the sensitivity of this analysis could distinguish two reaction rates only if they were different by more than a factor of 2. Very slowly reacting species would be excluded by the 12–14 min treatment period.

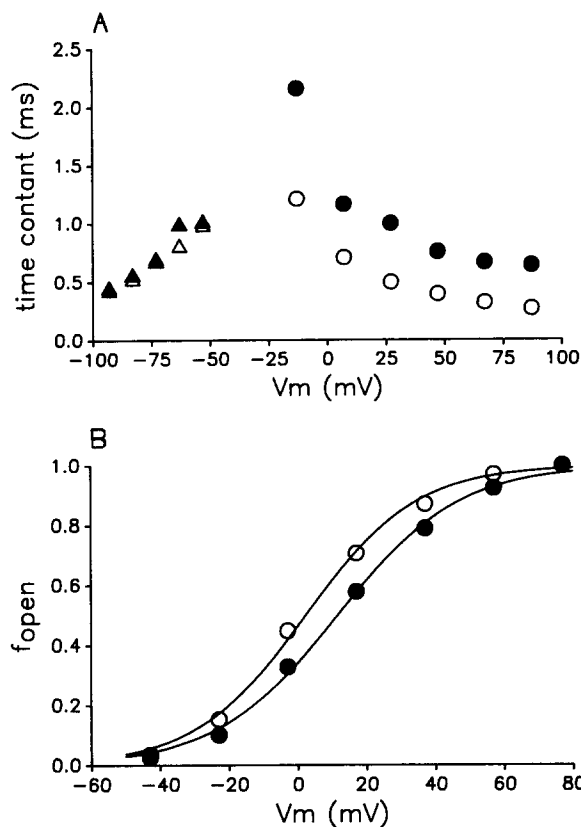


FIGURE 7. Actions of rose bengal treatment on K channels. *A*, Activation (circles) and deactivation (triangles) before (open symbols) and after (solid symbols) a 12-min treatment with $25 \mu\text{M}$ rose bengal in pH 7.5, 50 K ASW. *B*, Voltage dependence of the fraction of open channels before (open circles) and after (solid circles) rose bengal treatment. Solid lines represent the best fit of the Boltzmann function with midpoints of 2.3 and 11.6 mV and equivalent charges of 1.5 and $1.4e$ before and after treatment, respectively.

DEP and K Channel Gating Currents

Gating currents are produced by intramembranous charge movements; the largest gating currents are produced by fast, voltage-dependent conformational changes in the channel protein. We measured K channel gating currents before and after treatment with DEP to determine if the chemical groups modified by this reagent are involved in strong or weak voltage-dependent steps in the channel conformational change process. Fig. 9 illustrates the results of such an experiment.

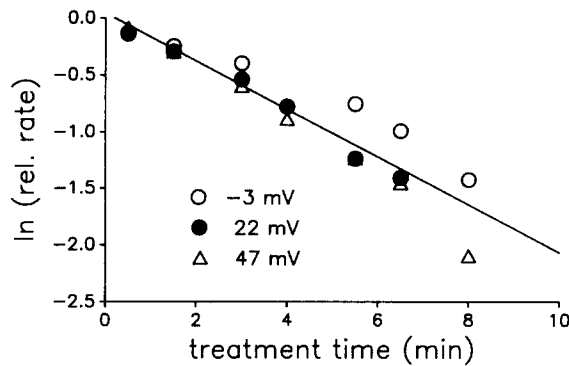


FIGURE 8. Rate of DEP modification of K channel kinetics. Time constants were obtained at the voltages indicated during treatment with $100 \mu\text{M}$ DEP in pH 6, 50 K ASW. The time constants slowed progressively during the first 9 min of treatment and were monitored for an additional 3-min exposure to DEP. The relative change in time constants at each of the three voltages was computed

and plotted in semilogarithmic form. A linear relationship is expected if DEP reacts with the target residues with a single rate constant.

The inset in Fig. 9 A shows K channel gating current recorded at -12 mV before (*top*) and after (*bottom*) treatment with $350 \mu\text{M}$ DEP in pH 6 Tris- NO_3 ASW. No large changes are evident. Indeed, the charge moved (A) obtained at several voltages is not significantly affected by this treatment. The solid lines are the fits of a Boltzmann function similar to that in Eq. 1 but scaled by the maximum charge moved. The parameters obtained from this fit show no appreciable effect of DEP. The midpoints of these relations are -34 and -32 mV with equivalent charges of 2.5 and $2.2e$ and

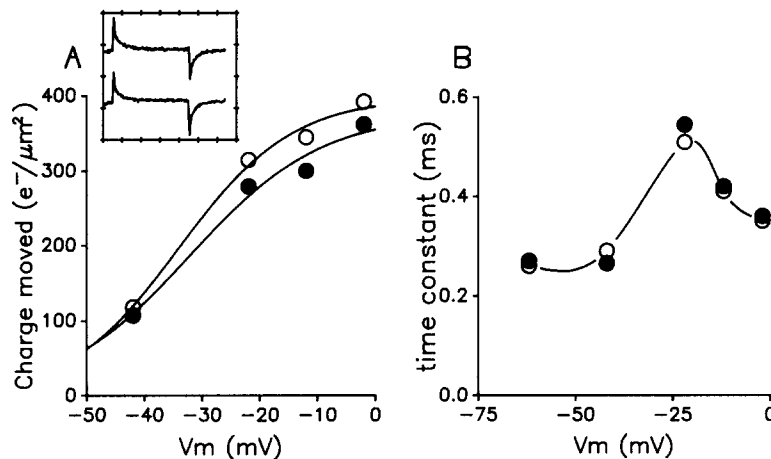


FIGURE 9. DEP and K channel gating currents. A, Voltage dependence of K channel gating charge movement before (open circles) and after (solid circles) a 4-min treatment with $350 \mu\text{M}$ DEP in pH 6, Tris- NO_3 ASW (21°C). The solid lines are the best fits of the Boltzmann function with midpoints of -34 and -32 mV , equivalent charges of 2.5 and $2.2e$, and maximum value of 398 and $377 e/\mu\text{m}^2$ before and after treatment, respectively. The inset shows the time dependence of K channel gating current recorded at -12 mV before (*top*) and after (*bottom*) treatment. Tick marks are at $25 \mu\text{A}/\text{cm}^2$ (ordinate) and 1-ms intervals. B, K channel gating current time constants before (open circles) and after (solid circles) treatment. The solid lines are cubic spline functions and are without theoretical significance.

maximum values of 398 and 377 $e/\mu\text{m}^2$ before and after treatment, respectively. These values are similar to those (average midpoint and equivalent charge of -38 mV and $2.2e$) obtained in our previous study of K channel gating currents (Spires and Begenisich, 1989). Fig. 9 B shows that gating current time constants were also unaffected by DEP treatment.

Fig. 10 summarizes the kinetic effects of DEP on ionic and gating currents. In this figure, the ratios of ionic or gating current time constants after DEP treatment to control values are plotted against membrane potential. Data from ionic currents are represented by open symbols and gating current data by solid symbols. Ionic current data at potentials negative to -40 mV are from K channel tail currents. Data from a total of 11 axons are plotted. Experiments with five axons provided data only on ionic currents, four axons provided only gating current data, and in two experiments

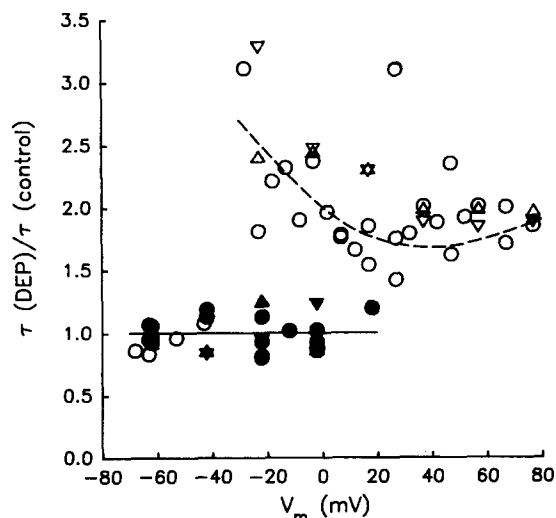


FIGURE 10. K channel ionic and gating current time constants. The ratio of ionic current (open symbols) or gating current (solid symbols) time constants after DEP treatment to control values are plotted. In each of two experiments (triangles and inverted triangles), both ionic current and gating current time constants were obtained. The open symbol data at potentials more negative than -40 mV are deactivation time constants. The solid line represents no effect of DEP. The dashed line is meant only to suggest a trend

in the data seen more clearly in individual axons (e.g., see Fig. 3 and the two individual experiments plotted as triangles in this figure). Data from 11 axons.

both ionic and gating currents were measured (triangles). In all but two experiments 0.35 mM DEP was used with treatment times of 4–11 min. We used 0.1 mM for 12 min in one experiment and 0.5 mM for 17 min in the other.

The data in this figure illustrate that DEP had little or no effect on K channel deactivation (tail) time constants or on K channel gating current kinetics. There was, however, a large slowing of the ionic current activation time constant. This slowing appeared to be somewhat voltage dependent (also seen in Fig. 3) and ranged from about a factor of 2.5 near -25 mV to about 2.0 at large positive potentials. Even at potentials where the gating and ionic current measurements overlap (-20 – 0 mV), the ionic current kinetics showed a two- or threefold slowing with no consistent effect on gating current kinetics.

There was also no large change in the maximum amount of gating charge moved.

In six experiments the ratio of the charge after treatment to control values was 0.83 ± 0.05 (SEM). Much of even this small reduction was due to several long experiments, especially the two in which both ionic and gating currents were measured. As described in Methods, our gating current solutions minimize but cannot eliminate the loss of K channel current that occurs with K-free perfusates. In the two shortest experiments the reduction of maximum charge moved was only 12%.

The solutions and temperature used for gating current measurements were quite different than those used for measurements of ionic currents and contained high concentrations of Tris (see Methods). Tris is known to accelerate decomposition of DEP (Berger, 1975), which could have accounted for the observed lack of effect of this reagent. We used three methods to examine this issue: (a) One of the gating current experiments included in Fig. 10 was done with 0.5 mM DEP for 17 min rather than the usual 4-min treatment with 0.35 mM. (b) In another gating current experiment included in the figure DEP treatment was done in the same solutions as used for ionic current measurements. (c) In two experiments (triangles) we exploited our ability to measure ionic currents after measurement of gating currents on the same axon. In these two experiments DEP was applied in the gating current solution and ionic currents were recorded before and after treatment. The data (triangles) in the figure show that while the ionic current kinetics were substantially slowed (by almost a factor of 3.5 at -20 mV), there were no effects on gating current kinetics.

DISCUSSION

Comparison to Earlier Results

There are some earlier reports of the actions of DEP and rose bengal on ion channels in axons. Shrager (1974) examined the effects of pH on K channel ionic currents in crayfish giant axons. The K channel activation kinetics are slowed by pH with an apparent pK_a near 6.3, a value similar to the pK_a of the imidazole group of the amino acid histidine. He found that DEP produced qualitatively similar effects on K channels but not on Na channels. Further, he reported unpublished observations of K channel effects by other histidine reagents including rose bengal. These results led to the suggestion that a histidine residue is involved in the "mechanism responsible for the control of potassium conductance" (Shrager, 1974).

Bookman (1984) reported that rose bengal slowed squid axon K channel activation kinetics with little effect on deactivation. Oxford et al. (1978) showed that internal application of 1 mM DEP for 30 min reduced Na channel current by only 27% with no noticeable effects on Na kinetics or inactivation.

We have added to these earlier studies a quantitative investigation of the actions of both DEP and rose bengal. External application of these two compounds produced identical effects on K channel ionic currents: a slowing of channel opening with no effect on channel closing rates and a shift of the voltage dependence of channel opening. The actions of DEP were specific for K channels and altered K channel activity only when applied externally. We extended this investigation to include K channel gating currents on which we found no detectable effect of DEP. We also

measured the time course of DEP slowing of activation time constants; these results were consistent with modification of a single kinetically distinguishable chemical group.

The effects produced by these histidine reagents are quite similar to those produced in K channels by external application of Zn^{2+} (Gilly and Armstrong, 1982). These authors suggested that divalent cations stabilize a nonconducting conformation of the K channel protein that undergoes a voltage-dependent structural change. They further suggested that the divalent cation binding site is formed by histidine groups on the K channel. More recently, Armstrong and Matteson (1986) showed that Ca^{2+} ions had an influence on K channel gating properties similar to that produced by Zn^{2+} , suggesting that their proposed mechanism may apply to physiological situations.

Functionally Important Histidine Residues

The conditions used for DEP and rose bengal application in this study were designed to maximize their specificity for histidyl residues (Lundblad and Noyes, 1984). While these two reagents can modify other amino acids, they have overlapping specificities only for histidine (Means and Feeny, 1971; Lundblad and Noyes, 1984). Consequently, our data are consistent with the suggestions of Shrager (1974) and Gilly and Armstrong (1982) that there is a histidine group (or several residues that react with DEP with indistinguishable kinetics) on the external surface of the K channel protein involved in the conformational change leading to the open state but not involved in channel closing. However, contrary to the model proposed by Gilly and Armstrong (1982), the lack of effect on K channel gating currents suggests that this conformational change is a weakly voltage-dependent process.

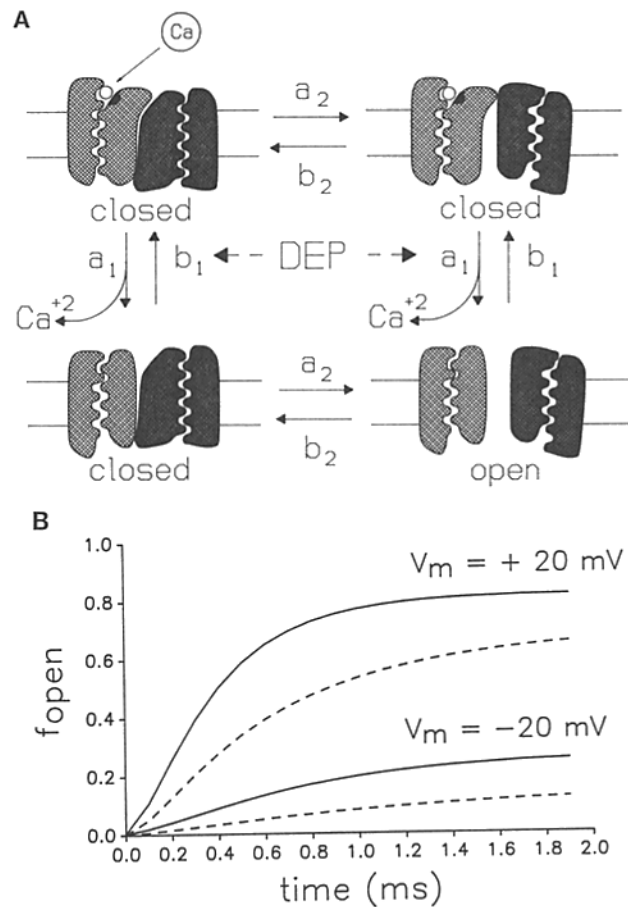
The amino acid sequence for the squid axon K channel has not been determined. Consequently, we cannot identify histidine residues on this molecule that might be candidates for the modifications we have produced. However, as described in the Introduction, sequences for several other K channels have been determined, including delayed rectifier and inactivating, A-type channels. The amino acid sequences of the delayed rectifier K channels from mouse brain (Tempel et al., 1988) and rat hippocampus (Christie et al., 1989) differ by only 3 of 495 amino acids. There are only nine histidine residues in these sequences. Depending on which protein folding pattern is assumed, there are either five (Tempel et al., 1987) or two (Catterall, 1988) histidine groups on extracellular loops. There are none in any of the putative membrane spanning regions H1 to H6. There is one histidine residue in the putative voltage-sensing region S4. Indeed, the predicted amino acid sequence for all K channels that have been cloned includes a histidine in the S4 region. Notably, none of the predicted Na or Ca channel sequences have histidines in their S4 regions.

As in squid axons, DEP modifies delayed rectifier in mouse neuroblastoma cells. Quandt (1988) showed that 1 mM DEP eliminates current through both fast and slow delayed rectifier K channels. Thus, histidine-modifying reagents alter the function of K channels in several different cells including those of mammalian origin. The histidine residues in the extracellular loops or the one in the S4 region may be reasonable candidates for mutagenesis.

A K Channel Kinetic Model

Any mechanism proposed to account for the actions of DEP on K channels must specifically address the following observations: (a) a slowing of the ionic current activation time constant with no measurable change in gating current or deactivation time constants, and (b) the shift of the activation curve to more positive potentials. This latter point is significant since DEP is a neutral molecule and its action reduces the positive charge associated with protonation. Consequently, a shift toward negative rather than positive potentials might be expected.

The differential effects of DEP on ionic and gating currents are qualitatively consistent with the ionic current activation time constant arising from a relatively slow, weakly voltage-dependent step in channel opening. Gating models for K channels based on a linear sequence of states cannot reproduce this observation. As described by White and Benzanilla (1985), purely sequential models require the slower, less voltage-sensitive steps to occur before the last transition to open channels. Therefore, any slowing of these slow steps by DEP will, necessarily, result in a slowing of gating current kinetics.



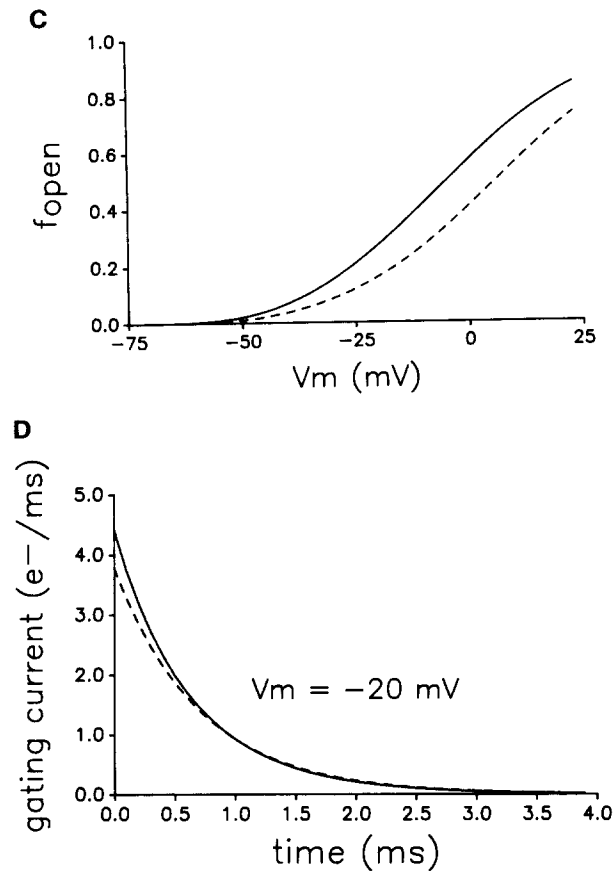


FIGURE 11. A K channel model and simulations of the actions of DEP. *A*, A simple kinetic model of K channels. Two types of independent subunits are depicted (light and dark shading), each of which can exist in two conformations. The ion pore is "open" only when both subunits attain the proper configuration. The action of DEP is assigned to the slow, weakly voltage-dependent transition as indicated. *B*, Time dependence of the fraction of open channels at two membrane potentials (-20 and 20 mV). Solid lines represent control conditions and dashed lines are after modification by DEP. *C*, Voltage dependence of fraction of open channels. *D*, Simulations of K channel gating currents. Parameters for control calculations (see Spires and Begenisich, 1989): a charge movement of $1.5e$ with a midpoint at -5 mV is associated with the transition between the two states of the lightly shaded subunit with time constants of 0.7 and 0.35 ms at -20 and $+20$ mV, respectively. The transition between the two states of the other subunit (dark shading) moves $3e$ (midpoint at -45 mV) with time constants of 0.42 and 0.2 ms at -20 and $+20$ mV, respectively. For the DEP calculations, the midpoint of the charge distribution of the slower subunit (light shading) was changed to $+6$ mV and the time constant values were doubled.

We have previously described a kinetic model for K channels derived to account for the measured properties of both ionic and gating currents (Spires and Begenisich, 1989). In this simple model the K channel is considered to be composed of two subunits, each of which can exist in two conformations. The details of this model can be found in Spires and Begenisich (1989) but its main features are depicted in Fig. 11 A.

Two different, independent subunits are illustrated by different shadings. Each subunit can exist in two conformations with transitions independent of the state of the other subunit. These transitions are voltage dependent due to the movement of charged (or dipolar) parts of the protein. The illustration follows that of Gilly and Armstrong (1982) in which positive and negative charges are at least partially paired. Application of a membrane voltage causes the charges to move relative to each other and this movement is ultimately coupled to the opening of the ion pore. This charge movement could be accomplished by a variety of mechanisms including "screw-like" motions of helical protein structures (Guy and Seetharamulu, 1986).

In our model we assigned relatively slow kinetics and a small charge movement (the equivalent of $1.5e$) to one subunit and faster kinetics with a charge movement of $3e$ to the other. The lightly shaded structure in the figure represents the slower, weakly voltage-dependent subunit; this model can account for the DEP data in a very natural way by ascribing all the effects of this reagent to a slowing of the transition between the two states of this subunit. A quantitative presentation of computation from this model is illustrated in the other panels of Fig. 11.

The model parameters for the computations shown in Fig. 11 are given in the figure legend. Only parameters governing the transition between the two states of the slower subunit were considered altered by DEP as indicated in the figure legend. Fig. 11 B illustrates the time dependence of the fraction of open channels which is proportional to ionic current. Computations with control parameters are plotted as solid lines; simulations of DEP effects are shown as dashed lines. The observed slowing of ionic current kinetics and the apparent voltage-dependent reduction of steady-state current are reproduced (cf. Figs. 1 and 2).

The observed voltage shift of the fraction of open channels is also duplicated by this model as illustrated in Fig. 11 C (cf. Figs. 6 and 7). The shift results from a DEP-induced change in the midpoint of the charge distribution of the slower subunit. The midpoint of this distribution occurs at the potential where $a_1 = b_1$. A decrease in the rate constant a_1 (with less or no effect on b_1) will produce a shift toward positive potentials since a larger depolarization would be needed for equality with the unchanged b_1 . A decrease in a_1 is also consistent with a slowing of the transition time constant.

This model accounts for the lack of effect of DEP on gating currents since (in terms of the model) these currents are dominated by the fast, voltage-dependent transition which is not considered to be affected by DEP treatment. This is presented in quantitative terms in Fig. 11 D.

It would be useful to be able to determine the quantitative changes in the model rate constants (a_1 and b_1) produced by DEP. However, the complexity of K channel behavior makes it difficult to obtain unique values for the individual model rate constants. Also, we have already described several limitations of this simple model

(Spires and Begenisich, 1989). These include an inability to predict the rising phase of K channel gating currents (White and Bezanilla, 1985; Spires and Begenisich, 1989). It is unlikely that K channels are composed of only two simple subunits; nevertheless, the success of this simple model in accounting for many of the significant features of K channel ionic and gating currents including the actions of histidine modification suggests that it may be worthwhile to continue to consider models consisting of some number of independent subunits.

As discussed above, one way to account for the similarity of DEP modification and the action of externally applied divalent cation action is to consider that the divalent cation binding site includes a histidine residue. This speculation is included in the model figure by the solid shading near the Ca ion. As suggested in Gilly and Armstrong (1982) and Armstrong and Matteson (1986), increased Ca^{2+} or added Zn^{2+} ions stabilize the nonconducting form of the channel. In terms of the model presented here, this is equivalent to a decrease in the a_1 rate constant, the same effect produced by histidine modification. Such considerations are clearly speculative but may serve to suggest experiments designed to investigate the functional roles of specific amino acids.

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