Different sites control voltage dependence and conductance of sarcoball anion channel

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ABSTRACT Single anion-selective channels from frog skeletal muscle SR were recorded using the sarcoball technique (Stein, P., and P. T. Palade. 1988. *Biophys. J.* 54:357–363). The voltage dependence of the open probability (P_o) was found to be dependent on the concentration of permeant anions on either side of the patch membrane. With 50 mM or greater permeant anions present on both sides of the membrane, the P_o vs. voltage plot yielded a bell-shaped curve centered around 0 mV (Hals, G. D., P. G. Stein, and P. T. Palade. 1989. *J. Gen. Physiol.* 93:385–410). When permeant anions in the bath (Cl^-) were replaced with relatively impermeant anions (gluconate, MOPS, propionate, or Hepes), the P_o vs. voltage relationship was shifted by ≈ -35 mV. Similarly, analogous experiments with the pipette solution produced a shift of comparable magnitude, but opposite polarity ($\approx +35$ mV). The stilbene derivative DIDS also shifted the voltage dependence, which suggests that amino groups may be involved in the shifts in voltage dependence. Other amino group modifiers reduced the single-channel conductance, and these data more strongly support the notion that amino groups are involved in conduction as well. The results indicate that amino groups involved in the conductance decrease are separate from those related to voltage sensitivity.

INTRODUCTION

The sarcoball technique is a new preparative recently developed in our laboratory, which enables direct patch clamp studies to be performed on channels from frog sarcoplasmic reticulum (SR) in native membrane (Stein and Palade, 1988). In a previous paper (Hals et al., 1989), a detailed report was given on the basic properties of the high-conductance anion channel found in these sarcoballs. These anion channels are highly conductive (505 ± 25 pS [$n = 35$] in symmetrical 200 mM TrisCl); steeply voltage sensitive with high open probability ($P_o = 0.95$) centered around 0 mV but falling to ≤ 0.05 by ± 25 mV; selective for small anions over small cations with $P_{Cl}/P_K = 45$, but poorly selective between smaller monovalent anions (Hals et al., 1989). The data in this paper were obtained from the same channel and sarcoball preparation.

This investigation began with the discovery that asymmetric changes in concentration of permeant anions modulate the sarcoball anion channel's voltage sensitivity. Specifically, lowering Cl^- concentration[1] on either the bath or pipette side of the patch produced a large shift (≈ 35 mV) in the P_o vs patch potential curve along the voltage axis. We hypothesized that selective anion neutralization of positive charges near the channel's voltage sensor may have produced the shifts in voltage dependent gating. Gating and selectivity of channels have traditionally been treated as independent processes (Hille, 1984) and only few reports have been published demonstrating interaction between these two aspects of channel function (e.g. Ciani et al., 1978; Chesnoy-Marchais, 1985; Nelson, 1986). We report here on the results of experiments that were performed in the presence of various amino group modifiers (DIDS,[2] SITS SPIT, TNBS, and pyridoxal phosphate) to neutralize the postulated positive charges. The results suggest that two classes of amino groups (accessible to the modifiers) may exist on the sarcoball anion channel; those involved in the shifts in voltage dependence, and those primarily involved in conduction. Some of these results have been presented previously in abstract form (Hals and Palade, 1989).

METHODS

Sarcoball production

Sarcoballs were produced from semitendinosus muscle of *Rana catesbeiana* according to previously described protocols (Stein and Palade,

[1]The asymmetric changes involved only monovalent anion replacements without substantial changes in cation concentrations or osmolarity.

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[2]*Abbreviations used in this paper:* DIDS, 4, 4′-diisothiocyano-stilbene 2, 2′-disulfonic acid; DNDS, 4, 4′-dinitrostilbene 2, 2′-disulfonic acid; TNBS, trinitrobenzene sulfonic acid; SITS, 4-acetamido, 4′-isothiocyano-stilbene 2,2′-disulfonic acid; SPIT, 3-sulfophenyl isothiocyante.

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1988). Briefly, a single muscle fiber is dissected and skinned mechanically with fine forceps in physiological saline, using a dissecting microscope. The skinned portion of the fiber is then transferred to the recording dish, filled with the desired recording solution. All of the data presented here were obtained from excised inside-out patches. Previous data on the sensitivity of Ca release channels found in sarcoballs indicate that in inside-out patches the cytoplasmic surface of the channels faces the pipette solution, and the SR luminal surface faces the bath solution (Stein and Palade, 1988). Protein modification and pH changes were performed in the bath solution. The details of the recording and bath perfusion systems used have been previously described (Hals et al., 1988). Potentials given are those in the pipette.

Solutions

The standard conditions for sarcoball recording were to begin in symmetrical solutions of 200 mM Tris-hydroxymethyl-aminomethane hydrochloride (TrisCl), adjusted to pH 7.0 with MOPS acid; modifications were then made to the bath solution after obtaining control records in each case. The anion substitutions were performed so that cation concentrations were maintained (i.e., cation equilibrium potentials remained at 0 mV) throughout each experiment. The bath recording solution also contained an additional 2 mM CaMOPS added to aid seal formation. Free Ca^{2+} concentrations in the pipette were not controlled but have been shown not to affect channel activity (Hals et al., 1988). Additional Tris base or MOPS acid was added to vary the pH of the bath solution. 4-Sulfophenyl isothiocyanate (SPIT) was obtained from Aldrich Chemical Co., Milwaukee, WI. 4,4'-Dinitro-2,2'-stilbene disulfonic acid (DNDS) was obtained from Pfaltz & Bauer Inc., Waterbury, CT. Tris base and MOPS were from Calbiochem-Behring Corp., La Jolla, CA. All other chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

Analysis

Single-channel amplitudes were measured either manually from the chart paper records or by computer. Data to be analyzed was played back through a model 902 LPF eight-pole Bessel filter (Frequency Devices Inc., Haverhill, MA) at 0.5 kHz (−3dB) and digitized at 500-μs intervals using a Data Translation Inc. (Marlboro, MA) DT2801A board and a PC's Limited IBM PC AT clone. The "Analysis" single channel analysis program developed by Dr. Hubert Affolter (courtesy of Dr. R. Coronado) was used to determine channel amplitudes by the following protocol: Single-channel amplitudes which fell into a user-defined window were obtained by subtracting the mean closed current from the mean open current. The open probability was calculated from the amplitude histogram by dividing the area of the "open" distribution by the summed area for both "open" and "closed" distributions, i.e., the total time. Open and closed time histograms were obtained using the IPROC2 single-channel analysis program (Sachs et al., 1982). In all cases, open probability measurements and time constants were taken from single-channel patches (i.e., where no multiple levels of current were ever observed during the entire experiment). The liquid junction potential produced by changing bath solution (for example) from 200 KCl to 200 mM KGluconate was measured as follows. An electrode filled with 200 mM KCl was lowered to the bath (also 200 mM KCl) and offsets were zeroed. The bath solution was changed to 200 mM KGluconate and the liquid junction potential was then measured in millivolts. Typically, junction potentials were in the range of 4–6 mV.

RESULTS

[Cl^-] modulates voltage dependence

In the process of measuring selectivity of the channel to a variety of anions (Hals et al., 1989), we observed that the voltage dependence of the channel was dependent on whether permeant anions were symmetrically or asymmetrically distributed. Specifically when bath Cl^- ions (or other equally permeant anions) were replaced with relatively impermeant substitutes (while maintaining cation equilibrium potentials at 0 mV), the P_o vs. patch potential curve was shifted along the voltage axis by ≈ -35 mV.

The shift in voltage dependence that results from total replacement of Cl^- ions in the bath by gluconate is illustrated in Fig. 1. A control P_o vs. voltage curve, occasionally observed to be slightly offset from 0 mV in symmetrical 200 mM KCl, is shown in Fig. 1 *A*. Current records after complete replacement of bath Cl^- by 200 mM gluconate are shown in Fig. 1 *B*. The reversal potential was shifted 43 ± 5 mV ($n = 5$), indicating that the channel was highly Cl^- selective, whereas regions of high open probability were shifted to negative potentials, as shown in Fig. 1 *C*. Such effects were reversible; when a different patch was moved back into symmetrical Cl^- solutions after the shift was complete, the P_o vs. voltage plot was again centered near 0 mV ($n = 2$; data not shown). The shifts in voltage dependence are in the opposite direction to the shifts in anion reversal potential produced, which renders a current-dependent mechanism for the shift less likely. Cations are also not likely to be a source of the shift, as 200 mM K^+ ions were present in both bath and pipette solution throughout the experiment. Further, equivalent results were obtained using Tris as the cation. With a constant concentration of 200 mM TrisCl in the pipette, the P_o was still centered around 0 mV with 100 mM TrisCl in the bath, but with 50 mM TrisCl in the bath, the P_o was shifted in the negative direction by −12 to −15 mV ($n = 4$; data not shown).

An analogous shift, but of opposite polarity, was obtained when Cl^- ions in the pipette were completely replaced by 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes). This effect is shown in Fig. 2, recorded from a patch with 200 mM TrisCl in the bath and 200 mM TrisHepes in the pipette. Compared to patches formed in symmetrical Cl^- solutions (control experiments in separate patches such as Fig. 1 *A*), open probability in the absence of pipette Cl^- was high at more positive potentials. The shift in peak open probability, after subtraction of the liquid junction potential, was ≈ +34 mV from the control value of 0 mV. The shift was

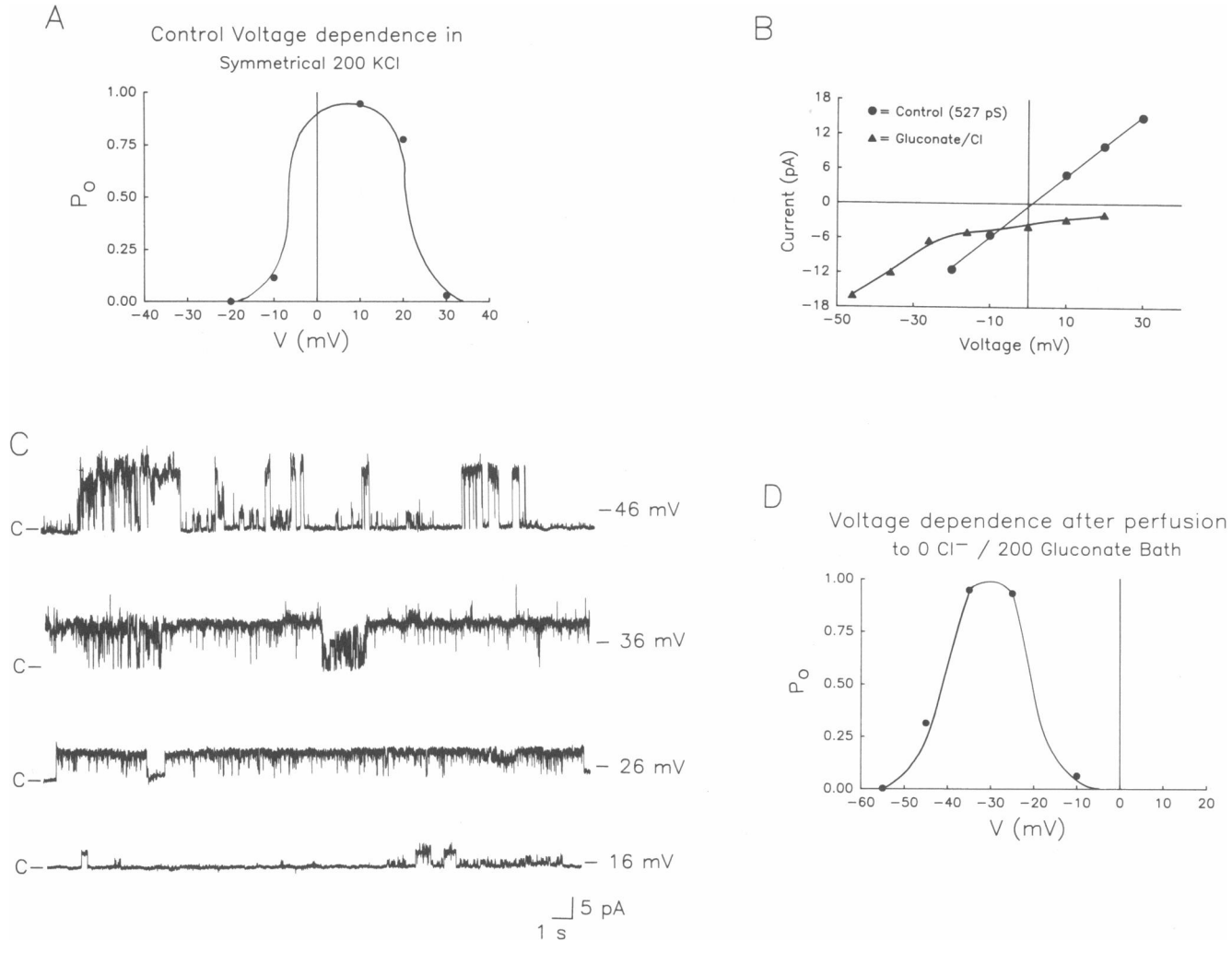

FIGURE 1 Replacement of bath Cl^- with 200 mM gluconate shifts anion channel voltage dependence. (*A*) Open probability plot for the patch in control solutions (symmetrical 200 mM KCl). (*B*) Current/voltage plots for the patch under control conditions (symmetrical 200 mM KCl, *circles*) and after bath perfusion to 200 mM KGluconate (*triangles*). (*C*) Data records from the same patch as in *A* after complete bath replacement of Cl^- ions with gluconate. The channels are again rarely open at −10 mV, but now the entire open probability curve is shifted in the negative direction by ≈ 40 mV. This shift is virtually equal (but opposite in polarity) to the amount of the shift for pipette replacement of Cl^- ions (≈ 34 mV). (*D*) The shifted voltage dependence apparent in the P_O vs. voltage plot after complete replacement of bath Cl^- ions with gluconate.

not specific for Hepes, as it was also present and of similar magnitude when gluconate or MOPS (3-morpholinopropanesulfonic acid) were substituted for Cl^- ions in the pipette (n = 5; data not shown).

Not all anion substitutes were capable of shifting the voltage dependence. Of all the anion substitutes tested (Hals et al., 1989; Table 1), only gluconate, Hepes, MOPS, oxalate, and propionate produced a simple shift in the P_o vs. voltage relationship. The least permeant anions (as determined from permeability rations [Hals et al., 1989]) produced the largest shift. Anions which are as permeant or more permeant than Cl^- (such as Br^- or HCO_3^-) produced no shift (Fig. 3). As shown in Fig. 3, the anion's permeability in the channel was found to be well correlated to the magnitude of the shift in P_o. Although permeability is roughly related to the anion's size, neither molecular weight nor hydrated radius correlated as well with the shift in voltage dependence as did anion permeability in the channel (Fig. 3, legend).

Based on these results, we hypothesized that the shifts in voltage dependence could be the result of interaction of permeant ions with charges near the channel's voltage sensors. If the charges were located near the selectivity filter or conduction pathway, then these charges might

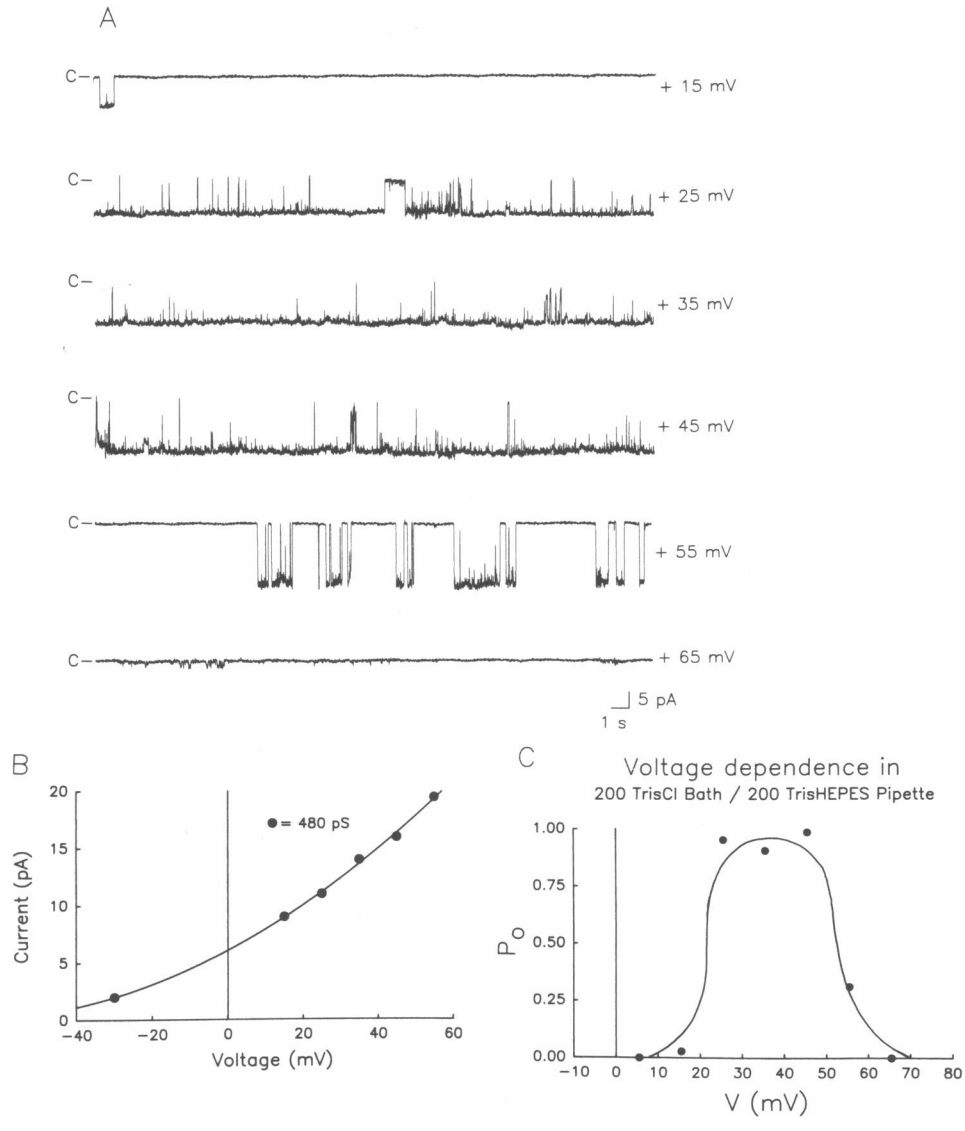

FIGURE 2 Shift in voltage dependence with total replacement of pipette Cl^- ions by Hepes. (*A*) Records used to generate the open probability plot in *C*. (*B*) Current/voltage plot for this patch. The conductance value given (480 pS) is measured on the linear portion of the data (> 30 mV). Data are from a single-channel patch recorded with 200 mM TrisHepes in the pipette. The junction potential was estimated by taking an electrode and zeroing the offset in symmetrical 200 mM TrisCl. The bath solution was then perfused to 200 mM TrisHepes and the offset at the electrode was measured at ≈ −4–5 mV. The potentials in *A* and *B* were corrected for this offset before data analysis.

also influence the channel's selectivity or single-channel conductance.

pH experiments

To help determine whether positive or negative charges were involved in the shifts and what amino acids might be associated with these charges, we performed experiments at different pH's. Varying the pH of the bath solution from 4.0 to 8.0 did not produce shifts in the voltage dependence (n = 3 at each pH; data not shown). Thus, if accessible positive charges are involved in the shifts in voltage dependence they would have to be titrated at pH values above 8.0. Because the P_K of amino groups is ≈ 9, we would have liked to performed experiments 9.0 or higher. Unfortunately, we were only able to raise pH to 8.0 as higher pH's caused the patches to become very leaky.

Whereas these changes in bath pH did not affect the voltage dependence of the anion channel, increasing bath

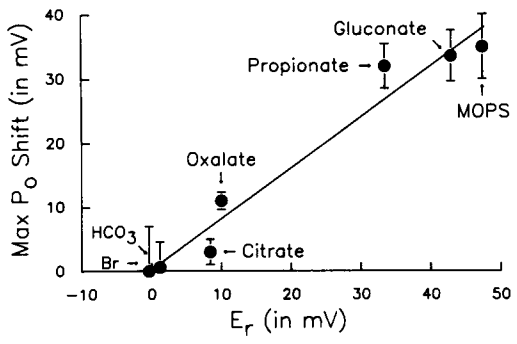

FIGURE 3 Shift in voltage dependence as a function of relative permeability of the anion substitute in the sarcoball anion channel. Plotting the data as a function of hydrated radius or molecular weight of the anion substitute did not produce correlations as good as that shown here for permeability. (P_o vs. reversal potential, correlation coefficient = 0.98; P_o vs. molecular weight, correlation coefficient = 0.52; P_o vs. hydrated radius, correlation coefficient = 0.18). Number of experiments/point: gluconate (6), MOPS (3), propionate (3), oxalate (2), Br (3), HCO_3 (2).

pH did have pronounced effects on its conductance. At bath pH of 8.0 the current-voltage relationship remained linear, but the conductance was reduced from 505 to 257 pS on either side of 0 mV (Fig. 4). The decrease in conductance was partially reversible; conductance was restored to near normal levels (435 pS) after returning the patch to the original pH 7.0 solution (n = 3, one example of which is shown in Fig. 4).

Protein modification

Whereas the pH experiments did not provide any evidence for or against involvement of positive charges in the shifts in voltage dependence, the results did suggest possible involvement of positively charged amino groups in conductance. Therefore, to further investigate involvement of positive charges (amino groups) in both voltage dependence and conductance, five amino group reagents were tested for their effects on the sarcoball anion channels: DIDS, SITS, TNBS, SPIT, and pyridoxal phosphate. Unless otherwise indicated, all modifiers were added to the bath solution only. Effects were grouped into three categories: shifts in voltage dependence, irreversible changes in conductance, and flicker block.

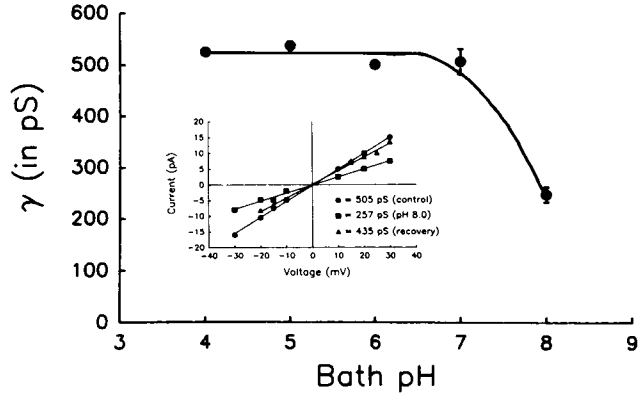

FIGURE 4 Conductance vs. pH of the bath solution. The only effect observed was a substantial decrease in conductance at a bath pH of 8.0 (247 ± 15 pS, n = 3). Current-voltage relations for the channel at pH 7.0 (control), pH 8.0, and after wash-out of bath solution to restore pH to 7.0 (recovery) are shown in the inset.

Shifts in voltage dependence

DIDS was the only amino group modifier tested that produced a shift in voltage dependence, but the shift was not observed immediately. After 8–15 min of exposure to DIDS in symmetrical Cl^- solutions,[3] a dramatic change occurred; the channels were no longer observed open in their normal potential range, the open events were much more brief, and the conductance was decreased. In two experiments, the unbound DIDS was extensively washed from the bath solution after these changes were observed and the effects of gating appeared to be irreversible (not shown). In comparison effects on conductance were reversible in two out of three wash-out attempts.

The records shown in Fig. 5 were obtained after removal of DIDS from the bath. The data traces in Fig. 5 *A* demonstrate altered channel gating: open events were much more brief and less numerous than in the control case (not shown). The maximal open probability was consistently decreased if the shift in voltage dependence had occurred due to modification by DIDS. The decrease in overall open probability most likely was a result of altered gating of the channel rather than a result of simple block of the open channel, as the decrease persisted after all unbound DIDS was removed from the bath.

The current records and the P_o vs. potential plot (Fig. 5 *B*) also demonstrate the shift in voltage dependence. While observed in only three of seven experiments attempted (Table 1), the shift of ≈ -30 mV is quantitatively similar to the shift produced when Cl^- ions are totally removed from the bath solution (Fig. 1 *C*). It is possible that some modification attempts were unsuccessful because the modification required at least several minutes of exposure to DIDS and several patches broke within 10 min of DIDS addition, perhaps before the reaction had a chance to occur. Addition of 660 μM DIDS to the pipette solution also had similar effects (the shift was in the opposite direction) (data not shown; n = 2).

To confirm that the shift in voltage dependence observed was due to protein modification by DIDS, the

[3]Using K^+ as the cation instead of Tris (a primary amine) did not significantly alter the time course of the modification (data not shown).

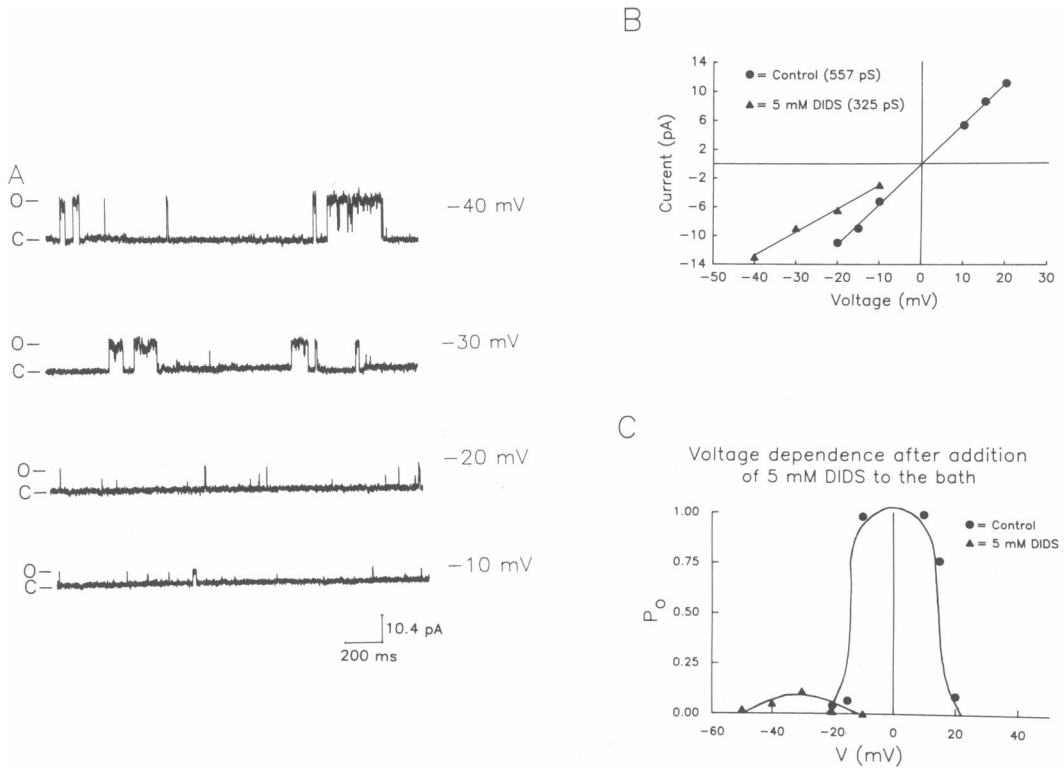

FIGURE 5 Shift in voltage dependence by DIDS. The records shown in *A* were obtained from an excised patch in symmetrical 200 mM TrisCl after 10–20 min exposure of a patch to 5 mM DIDS. (*A*) Events ≈ 15 min after the addition of 5 mM DIDS to the bath solution (SR luminal surface). Before DIDS addition, and even for many minutes after DIDS addition, events were not observed at these potentials. (*B*) Current/voltage plot for the control case (*circles*) and for the DIDS modified events (*triangles*). (*C*) Plot of open probability vs. patch potential from the data in *A* illustrating the shift in voltage dependence observed.

related compound DNDS was tested. DNDS has DIDS' reactive isothiocyano groups replaced with nonreactive nitro groups, so DNDS should reproduce any effects of DIDS on the channel not caused by DIDS' action as a protein modifier. DNDS added to the bath solution caused a flicker block of the channel's open events but no effects on the voltage dependence or maximal open probability of the channels were observed (n = 6; data not shown).

Irreversible reduction in conductance

Both SPIT and TNBS irreversibly reduced the conductance of the sarcoball anion channels by nearly one-half, without affecting their voltage dependence. An example of the effect of SPIT is shown in Fig. 6. Conductance was reduced from 511 pS in the control case, to 230 pS in the presence of 2 mM SPIT (Fig. 6 *B*). The reduction in amplitude of the events was observed 2–3 min after the addition of the modifier.

As seen in Fig. 7, 200 μM TNBS added to the bath solution also reduced the conductance to a similar extent (400 to 194 pS). The reduction in conductance by TNBS was uniform on both sides of 0 mV, and the change occurred < 1 min after the addition of TNBS. Removal of free TNBS from the bath did not bring the conductance back to normal (Fig. 7 *E*). None of the other amino group modifiers had any consistent irreversible effects on anion channel conductance.

Flicker block

We use the term "flicker block" to refer to the action of a blocking molecule that binds only weakly to a channel. The transient nature of the binding leads to a characteristic flickering of open events, in some cases seen as a decreased conductance when the rates of binding and unbinding exceed the recording bandwidth.

All of the modifiers used possess negatively charged groups: DIDS and SITS each have two sulfonate groups, SPIT and TNBS each have one sulfonate group, and the phosphate groups on pyridoxal phosphate have two negative charges. Except for TNBS, the negative charges are completely independent of the amino-reactive group on the modifier. Thus it is not surprising that varying degrees of flicker block were also observed in the presence of most

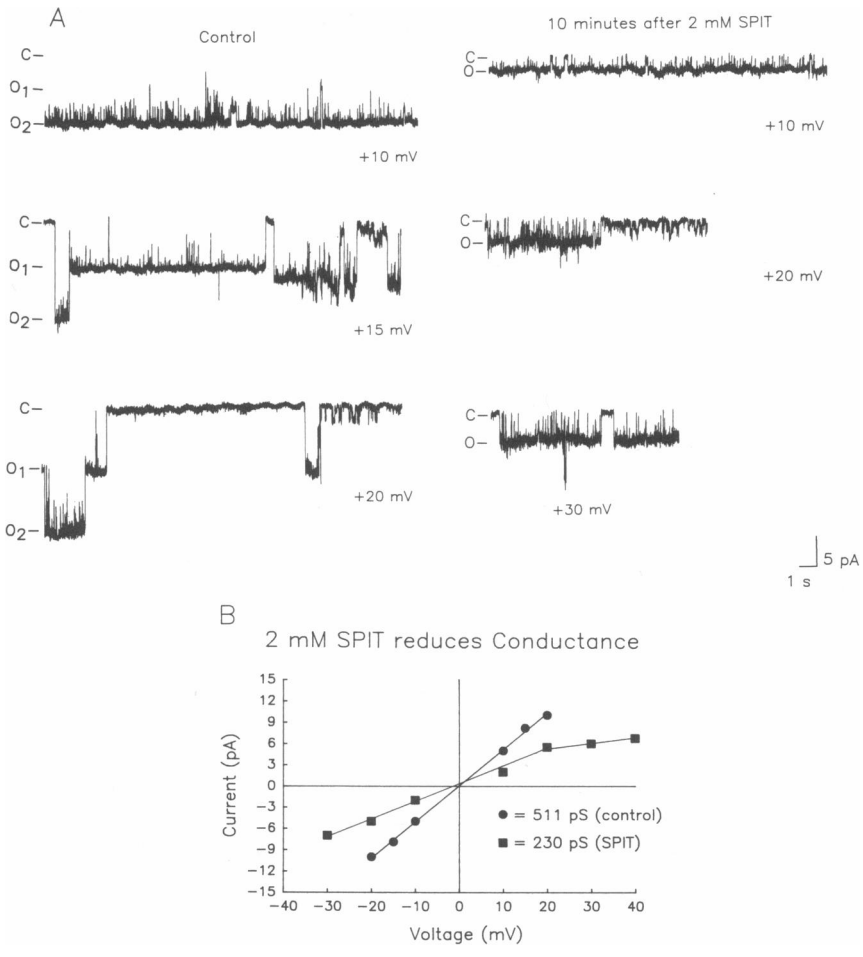

FIGURE 6 SPIT decreases the conductance of the channels. (*A*) Current records before (on the left) and after (on the right) addition of 2 mM SPIT to the bath solution. Note the decreased current amplitude after exposure to SPIT. These data are from an excised inside-out patch formed in symmetrical 200 mM TrisCl. (*B*) Current vs. voltage plots for the records shown in *A*. Note the decrease in conductance in SPIT. The slope conductance of 230 pS refers to that over the potential range from −20 to +20 mV; at more positive potentials, conductance is reduced further.

modifiers. These flicker blocks were removed after perfusion of the modifier from the bath solution. Flicker block probably contributed to reversible conductance decreases observed with DIDS, SITS, and DNDS.

An example of flicker block observed in the presence of TNBS is shown in Fig. 7 (apparent as increased noise during the channel openings as well as increases in resolvable brief closings; Fig. 7 *C*). Washing TNBS from the bath removed the flicker block (Fig. 7 *D*). The S1 state labeled on Fig. 7 *A* is an example of a previously described substate (Hals et al., 1989). Gating to the S1 state was initially frequent, but was greatly reduced before addition of TNBS.

A flicker block was also observed immediately after 1 mM SITS addition to the bath, and, as with the other modifiers, the flickers disappeared after the unbound SITS was washed form the bath ($n = 2$, data not shown). The slope conductance was reduced from 517 to 372 pS by 1 mM SITS, and washing unbound SITS from the bath solution restored conductance to 542 pS (not shown).

Because DIDS is a large negatively charged molecule and is known to have effects on several different anion channels (Miller and White, 1984; Hanrahan et al., 1985; Gray and Ritchie, 1985) and carriers (Ship et al., 1977; Lepke et al., 1976), it is not surprising that DIDS also had effects other than on the voltage dependence of the sarcoball anion channels. A flicker block was also observed immediately after the addition of 5 mM DIDS to the bath, and the slope conductance was reversibly reduced from 557 to 400 pS, with recovery to 548 pS after DIDS washout (not shown). Additionally, the two open time constants were reduced from 17 and 324 ms in the control case (at +10 mV, in symmetrical 200 mM TrisCl;

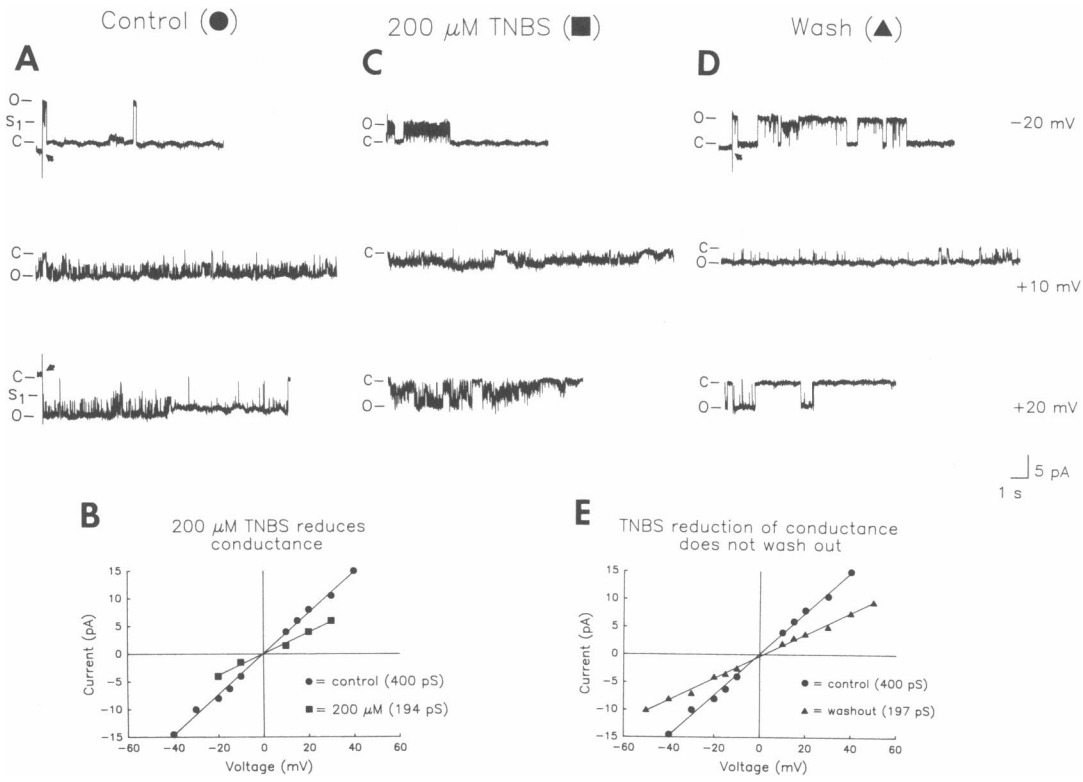

FIGURE 7 TNBS also decreased the conductance of the channels. An excised inside-out patch containing a single sarcoball anion channel is shown exposed to three different bath solutions; control (symmetrical 200 mM TrisCl), 200 μM TNBS (dissolved in 200 mM TrisCl), and wash back to control conditions. The corresponding current/voltage relationships are shown in the plots in *B* and *E*. The apparent "flickering" observed in the control records is a rare example of bursts of the S1 substrate in an excised patch (Hals et al., 1989), and is not related to drug addition. Note the presence of TNBS in the bath induces a rapid flicker block of the open events, which obscures some of the channel openings (7C), and that this flicker block is reversed when all unbound TNBS is washed from the bath solution (7D). The current/voltage relationships demonstrate a reduction in slope conductance (400 → 194 pS, 7C), and also show that the conductance does not recover (197 pS, 7E) when TNBS is washed from the bath.

Hals et al., 1989) to 6.3 and 30.2 ms, respectively, in the presence of DIDS (n = 1; data not shown).

The effects observed on the sarcoball anion channels as a result of the various amino group modifications attempted are summarized in Table 1. "Reactive group" refers to the portion of the modifier molecule that is involved in the actual amino group reaction. The numbers listed in the columns on the right of the table refer to the number of times the event was observed. The total number of experiments performed with each modifier is listed under *N*.

To summarize briefly, of the five modifiers tested only DIDS caused a shift in voltage dependence of the channels. SPIT and TNBS caused a large irreversible reduction in conductance, whereas pyridoxal phosphate had no consistent effect on the channels. Some modifiers (DIDS, SITS, and TNBS) also produced an obvious flicker-type block of the open events. The flicker blocks observed were reversible and therefore not related to any covalent modifications by these compounds.

DISCUSSION

Modifiers which had substantial irreversible effects on conductance (SPIT, TNBS) did not affect voltage dependence, and modifications by DIDS which did shift the voltage dependence were not usually associated with irreversible reductions in conductance. Thus, any amino groups associated with conduction are likely to be separate from those related to the shifts in voltage dependence.

Conductance changes

All the modifiers utilized were capable of neutralizing positively charged amino groups which might attract high local concentrations of anions in their vicinity. The decrease in current at all potentials after SPIT or TNBS modification is more suggestive of modification of a group in the narrow pore of the channel than groups at the

TABLE 1 Summary of protein modifier experiments

Modifier [ ]	Reactive group	N	Shift voltage dependence (Irrev.)	γ decreased (Irrev.)	Flicker block (Rev.)
DIDS (1–5 mM)	NCS	7	3 (−30 ± 1.5 mV)	1 (302 pS)	7
SITS (0.6–1 mM)	NCS	6	0	1 (370 pS)	6
DNDS (2 mM)	—	6	0	0	6
SPIT (1.5 mM)	NCS	5	0	5 (226 ± 77 pS)	1
TNBS (0.5 mM)	SO_3	4	0	4 (271 ± 89 pS)	4
Pyridoxal phosphate (0.8 mM)	CHO	6	0	1	0

Numbers in table not in parentheses refer to number of experimental observations. N refers to the total number of experiments performed with a given modifier. Rev. = Reversible; Irrev. = Irreversible.

channel mouth or in the vestibules. Modifications were performed on only one side of the channel, which should have had the result of decreasing only that vestibule's Cl^- concentration, reducing the current flowing from that side of the chennel, but not that from the opposite direction. Similarly, any positive charges being titrated at pH 8 were also unlikely to be vestibule-associated because titration of such charges in only one vestibule would not be expected to yield the linear current-voltage relationship that was observed. In this situation as well, two possibilities appear more likely: an effect on an amino group in the narrow pore portion of the channel (as previously mentioned above), or preferential activation of a substate instead of the main open state. If the substate explanation is correct, the substate in question could be the S1 state, by virtue of its similar conductance (Hals et al., 1989). Our results do not permit us to distinguish between these possibilities.

Shifts in voltage dependence

The observation that DIDS could irreversibly shift the channel voltage dependence when added to the bath solution is compatible with the hypothesis that amino groups may also be involved in the shifts in voltage dependence. The supposition that amino modification by DIDS, and not some other aspect of the molecule, was responsible for the shifts in voltage dependence is supported by several results. First, the reversible nature of the flicker block indicates that the sulfonates on DIDS and DNDS were only responsible for the flicker block and were not involved in the shifts in voltage dependence. Secondly, the shift in voltage dependence induced by DIDS was irreversible (and therefore taken as covalent), because total removal of unbound DIDS from the bath solution had no effect on the modified voltage dependence. Lastly, the modification of the voltage dependence by DIDS took several minutes to become apparent. Previous studies with the band 3 transporter from erythrocytes demonstrated that covalent binding (isothiocyano groups) of DIDS to band 3 protein took several minutes to occur, but noncovalent binding occurred immediately (Lepke et al., 1976; Ship et al., 1977).

The reasons why DIDS was the only modifier which shifted the voltage dependence remain a matter for speculation. DIDS is the only modifier used that has two amino-modifying groups per molecule and can therefore act as a cross-linker (perhaps a necessary step to cause the shift in voltage dependence). This could explain why SITS, a structurally similar but only monofunctional molecule, did not shift the voltage dependence. Alternatively, a higher SITS concentration or exposure for longer periods of time might have been required to shift the voltage dependence.

Hypotheses for the shift in voltage dependence by Cl^- ions

We have yet to arrive at a fully satisfactory model for the shift in voltage dependence induced by anion substitutions. Any plausible hypothesis would have to account for a sizable shift ($\approx$ 35 mV) in *both* limbs of the P_o vs. patch potential relationship observed when Cl^- ions were replaced by a relatively impermeant substitute, why the shift was opposite to the shift in reversal potential, why symmetric shifts could be produced by Cl^- replacement on either side of the membrane, and why the relative permeability seemed to be correlated with the magnitude of the shift observed (Fig. 3). In a polar subunit hypothesis which gave rise to a high P_o centered around 0 mV

(Ehrenstein et al., 1978), voltage applied caused the subunits to move and occlude the channel pore. Whereas interaction of certain ions with subunits on one side of the membrane could influence one of the limbs of the P_o-V relationship, it would seem unlikely to shift both limbs in the same direction.

Hypotheses involving interactions between anions and charged groups in channel vestibules also have their shortcomings. Selective attraction of impermeant anions into vestibules (Dani, 1986) containing positively charged amino groups would induce a local negative potential on the side with the impermeant anions. To offset this negative potential, an equivalent positive potential would have to be applied to that side of the membrane bathed in the Cl^- substitute. This would predict a shift of voltage dependence in the opposite direction to the shift in reversal potential, as observed experimentally, but then the correlation between permeability and the shift in voltage dependence (Fig. 3) would be merely fortuitous. If a conducting channel had its permeant anion-containing vestibule depleted of anions due to the channel's high conductance, together with a simultaneous accumulation of anions in the vestibule on the "impermeant" ion side of the membrane, the shift in voltage dependence would also be in the correct direction and might predict transient activation and time-dependent diminution of single channel current at certain potentials, both dependent upon the rate of depletion of permeant ions. Such decreases in single-channel current were not observed during transient channel activation (see Fig. 1, Hals et al., 1989).

The correlation between permeability and shift in voltage dependence could by itself be explained if a permeant ion needed to cross a hypothetical "selectivity filter" in the middle of the channel in order to bind to a site in the channel closer to the side bathed with the impermeant anions. Such a model would then predict the observed shift in voltage dependence only if the equivalent binding site on the other side were not occupied by a permeant ion, a proposition which also seems unlikely. Consequently, one of our most intriguing observations, the large shift in voltage dependence that occurs with anion substitution, remains to be fully explained. This observation nevertheless clearly demonstrates that channel gating and selectivity cannot be regarded as wholly independent processes in certain channels.

Physiological implications

Even though the concentration of myoplasmic Cl^- and other permeant anions in frog skeletal muscle is low (each $\leq$ 10 mM: Macchia et al., 1978; Vaughan-Jones, 1982; Godt and Maughan, 1988), a steep (albeit somewhat variable) voltage dependence, similar to the one observed in symmetrical 200 mM TrisCl, is observed when recording under a first approximation to physiological conditions (symmetrical: 5 mM Cl^-, 5 mM PO_4^{2-}, 10 mM HCO_3^-; data not shown).

It seems highly unlikely that the shifts in voltage dependence, the decreased P_o or the flicker block produced by DIDS here, even taken together, could account for the extent of inhibition of anion permeability observed by others. DIDS and SITS have previously been utilized to inhibit rabbit SR anion permeability, exhibiting apparent K_d's or 5 and 40 μM, respectively, in inhibiting SO_4^{2-} permeability (Kasai, 1981). DIDS was also shown to inhibit phosphate efflux from SR with an apparent K_d of 3 μM (Campbell and MacLennan, 1980). The inhibition of Cl^- conductance observed here was considerably less. This comparison suggests that there is a separate more DIDS- and SITS-sensitive anion transport system in isolated rabbit SR, and possibly also in frog SR. Reports of effects of DIDS on smaller conductance rabbit SR Cl^- channels in bilayers clearly state inhibition (Rousseau et al., 1988), although effects were not always complete (Smith et al., 1986). While we do not yet know whether these large conductance Cl^- channels exist in rabbit sarcoballs,[4] it seems likely that the density of this other transport system must be such that it can support greater fluxes of phosphate and sulfate than the chloride channel under study here. That so little of the anion permeability remained unaffected by DIDS (Kasai, 1981) might be compatible with our estimate that only ~1% of isolated SR vesicles would be likely to contain this Cl^- channel (Hals et al., 1989).

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[4]We have, however, observed less voltage-dependent high-conductance chloride channels in patch-clamped giant liposomes into which isolated rabbit SR vesicles had been incorporated by freeze-thaw (Hals et al., 1989).

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