Biochimica et Biophysica Acta, 514 (1978) 137–144

BBA 78197

THE EFFECT OF A DISULFONIC ACID STILBENE ON PROXIMAL CELL MEMBRANE POTENTIAL IN *NECTURUS* KIDNEY

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(Received December 12, 1977)
(Revised manuscript received April 18th, 1978)

Summary

The effects of 0.5 mM 4-acetamido-4′-isothiocyano-stilbene-2,2′ disulfonic acid on the electrical properties of the peritubular membrane were studied in the proximal tubule of the perfused *Necturus* kidney. The addition of stilbene isothiocyanate disulfonic acid in peritubular perfusate resulted in an average 4.5 mV hyperpolarization with no detectable changes of peritubular membrane input conductance. The depolarization elicited by high-K media was enhanced by 18% in the presence of stilbene isothiocyanate disulfonic acid, an observation indicating that the inhibitor increased the contribution of potassium to membrane potential, presumably by decreasing anionic permeabilities. The hyperpolarizing effect of stilbene isothiocyanate disulfonic acid was abolished when peritubular bicarbonate was removed from the medium and isoosmotically replaced by chloride. These data suggest that (a) intracellular bicarbonate activity is higher than that predicted for passive distribution, (b) stilbene isothiocyanate disulfonic acid decreases P_{HCO_3}, thus hyperpolarizing the membrane, (c) chloride distribution appears to be passive when bicarbonate is removed from the peritubular perfusate. The state of Cl distribution when extracellular bicarbonate is at physiologic concentration cannot be assessed from the present data.

Introduction

The early determinations of intracellular chloride concentration in proximal tubular cells of *Necturus* kidney yielded an average value of 32 mequiv./kg cell water [1] which is significantly higher than the activity of 4.7 mequiv./kg H_2O, required for electrochemical equilibrium (membrane potential difference, E_m,

Abbreviation: SITS, stilbene isothiocyanate disulfonic acid.

taken as −70 mV). Subsequent electrometric determinations of intracellular chloride activity $[Cl]_i$ by Khuri et al. [2] and by Spring and Kimura [3] confirmed the findings of the earlier workers: $[Cl]_i$ was found to be two to four times greater than that predicted for passive distribution. On the other hand, Khuri et al. have also reported [4] that the intracellular bicarbonate activity $[HCO_3]_i$ is about ten times higher than the theoretical value corresponding to equilibrium. The resulting bicarbonate equilibrium potential, E_{HCO_3}, of nearly 0 mV is far away from the E_m of about −70 mV. In another study [5], however, on the proximal tubule of bullfrog, $[Cl]_i$ was assessed by means of Cl-sensitive microelectrodes and was found to be passively distributed throughout its reabsorptive process.

Although species differences between amphibians may account for the observed discrepancies in $[Cl]_i$ estimates, one should not overlook the pitfalls inherent in electrometric determinations. It is well known for example that the sensitivity of chloride electrodes for chloride over other anions is not perfect; yet the distortion due to intracellular organic anions is difficult to assess. On the other hand, cell impalement may damage the electrode tip and alter its sensitivity slope, especially in tissues surrounded by a tough capsule like the kidney of Necturus. Such artifacts, if present, may produce erroneously high $[Cl]_i$ readings. Thus, we have used an independant, yet indirect electrophysiological technique, to investigate the state of anion distribution across the peritubular membrane in proximal cells of *Necturus* kidney.

4-Acetamido-4′-isothiocyano-stilbene-2,2′-disulfonic acid (SITS), an amino-reactive agent interacting with amino groups of cell membranes, was reversibly added into the peritubular perfusate, while recording membrane potential. SITS is a known inhibitor of anion fluxes in erythrocytes [6,7]. This agent has also been shown to block the Cl efflux and HCO_3 uptake in squid axon [8] and snail neurones [9] and to inhibit the anion-dependent part of the short circuit current across the turtle bladder [10]. In rat proximal tubule, SITS inhibits the H^+-glycodiazine (buffer)-dependent transport when applied from the peritubular side alone [11]. In the present study we have anticipated that SITS would depress anion permeability across the peritubular membrane in Necturus proximal tubular cells, as in other tissues. Since intracellular chloride [2,3] and bicarbonate [4] activities are believed to be significantly higher than those predicted for passive distribution, a decrease of anion permeability by SITS should shift membrane potential difference (p.d.) in the hyperpolarizing direction. It should be stressed that a negative result (no change in p.d. during exposure to SITS) may indicate either a lack of effect of the inhibitor on anion membrane permeability in *Necturus* or an equilibrium distribution for anions. On the contrary, an increase of intracellular negativity would strongly suggest that SITS does depress the permeability of the membrane to anion(s), the distribution of which is not consistent with electrochemical equilibrium.

Methods

All experiments were carried out in the doubly perfused *Necturus* kidney. The details for anesthesia and preparation of the animals were described elsewhere [12]. The control perfusing solution was a 82 mM NaCl solution, con-

taining in addition the following compounds (in mM): 13.0 $NaHCO_3$, 4.5 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$ and 2.2 glucose. Polyvinyl pyrrolidone (20 g/l) was also added to provide some oncotic force. In the experimental solutions 0.5 mM SITS was added (purchased from ICN Pharmaceutical Inc., Cleveland, Ohio, or from BDH Chemicals Ltd., Poole, U.K.). The tissue was reversibly exposed to the test solution by switching a stop-cock placed on the path of the caudal vein catheter. In some experiments high-K solutions were also used (45.0 mM KCl instead of 4.5 mM, the concentration of NaCl being reduced accordingly). In other experiments $NaHCO_3$ was reversibly removed and isosmotically replaced by NaCl, the buffer being provided by addition of approx. 5 mM Tris-maleate and 6.8 mM NaOH. The substituted solutions were used as such or supplemented with 0.5 mM SITS. Fresh solutions were prepared every day. Their pH was measured and, when necessary, adjusted to 7.4 by adding small amounts of HCl/NaOH. The solutions containing SITS were protected from sunlight during their preparation and throughout the experiment.

The methods for continuous recording of peritubular membrane p.d. and cell membrane input conductance have been described in detail elsewhere [13,14]. In the experiments in which bi-ionic potentials were performed (K/Na or Cl/HCO_3 substitutions) it was generally assumed that if a step change in the concentration of a given ion produces a step change in membrane p.d., then the permeabilities of the two ions involved in the substitution (K and Na or Cl and HCO_3) are not identical. In a few experiments oil was injected into the lumen of studied tubules, before impalement. Since the responses were similar, irrespective of luminal injection of oil, the data were pooled. The results are given as mean ± S.E.

Results

The effects of SITS on membrane p.d. The addition of SITS in peritubular fluid (10—40 s) invariably hyperpolarized the cell membrane. In the first series of experiments, in which we tested the effect of SITS alone, the average change in p.d. was -4.5 ± 0.4 mV, $n = 17$ ($P < 0.001$). The effect was essentially reversible, although the recovery was not always complete. On repetitive exposures of the tissue to SITS within a short lapse of time (e.g. 5 min) the magnitude of hyperpolarization tended to become smaller and smaller, presumably owing to the cumulative effect of the non-reversible fraction of the response of the tissue to SITS. Thus, the above mean was computed from the experiments (responses of the tubule to SITS) in which the tissue was not previously exposed to the drug for at least 20 min.

The effect of high-K media. The above observations strongly suggest that the application of SITS in peritubular fluid reversibly decreases the permeability of the membrane to one or several anions. The equilibrium potential for such anions A, E_A, ought to be less negative than membrane p.d., E_m, so that upon depression of their permeabilities by SITS, E_m can move in the opposite direction (it becomes more negative). If so, the relative contribution of potassium to membrane p.d. should increase during exposure of the tissue to SITS. To assess whether this readily occurs, the effect of high-K media on membrane p.d. was studied before and under application of SITS, in 7 single tubules. The amplitude of potassium-induced depolarization observed during exposure of

the tissue to SITS ($\Delta V_{K45,SITS}$) was compared to that previously recorded at the same tubule without the inhibitor ($\Delta V_{K45,cont}$) and expressed as a ratio. In each experiment $\Delta V_{K45,cont}$ was smaller than the corresponding $\Delta V_{K45,SITS}$. The normalized ratio $\Delta V_{K45,SITS}/\Delta V_{K45,cont}$ was on the average 1.18 ± 0.05, a figure significantly different from unity ($P < 0.01$).

The magnitude of depolarization elicited by high-K media ($\Delta V_{K45,cont}$), taken from 22 experiments, was on the average 29.1 ± 1.7 mV. This figure is similar to that reported previously by Boulpaep [15] and ourselves [16]. The half-time of depolarization was 2.2 ± 0.2 s, when the half time of repolarization (i.e. during recovery from high-K media) was 5.2 ± 0.6 s. Disparities between half-times of depolarization and repolarization after exposure to high-K media have been observed in several tissues. However, since the pioneer work of Hodgkin and Horowicz on that field [17], not much progress has been accomplished as to the underlying mechanisms. The two hypotheses advanced by these authors with regard to muscle fibres are pertinent to *Necturus* proximal tubule: 1. Appreciable quantities of K may be retained in a small compartment, lining the plasma membrane (the T-system in the muscle, the intercellular spaces in proximal tubule) and/or 2. The asymmetry may be connected with rectifying properties [18] of the potassium channel.

The effects of bicarbonate-free media. So far it has been established that SITS reduces the permeability of the membrane to some ions, presumably HCO_3 and/or Cl, thus indirectly increasing the transference number of potassium, T_K. To obtain more information on the relative inhibition of P_{Cl} and P_{HCO_3} by the drug, the effect of SITS was studied in six single tubules before and after removal of bicarbonate from peritubular fluid. The hyperpolarization elicited by SITS was −6.5 ± 0.5 mV when bicarbonate was present in the perfusing solution. It fell to an insignificant −0.2 ± 0.4 mV after chloride was substituted for bicarbonate. The difference is highly significant ($P < 0.001$). One representative experiment is shown in Fig. 1.

In the present, as in a previous, study [19], substitution of chloride for bicarbonate in peritubular fluid resulted in a large depolarization. It was suggested then that the removal of bicarbonate produced depolarization through

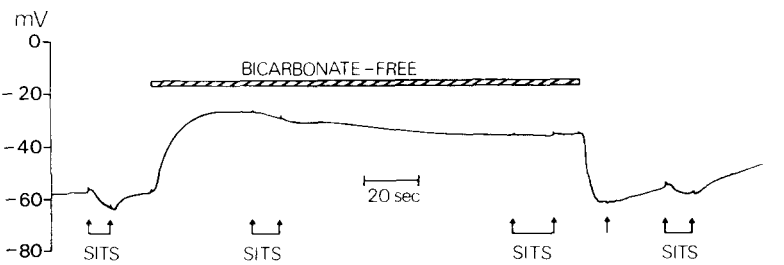

Fig. 1. Effects of SITS on cell membrane p.d. before and after substitution of chloride for bicarbonate in peritubular fluid. Withdrawal of bicarbonate results in 30 mV depolarization, as previously reported [19]. The addition of SITS produces hyperpolarization when HCO_3 is present in the solution, not when this anion is removed. Note that the magnitude of the response to SITS is slightly decreased after repetitive exposure of the tissue to the inhibitor (compare the hyperpolarization obtained at the first and fourth exposure). Single arrow indicates the beginning of a spontaneous decline of cell p.d., probably related to small motions of the tissue damaging the membrane.

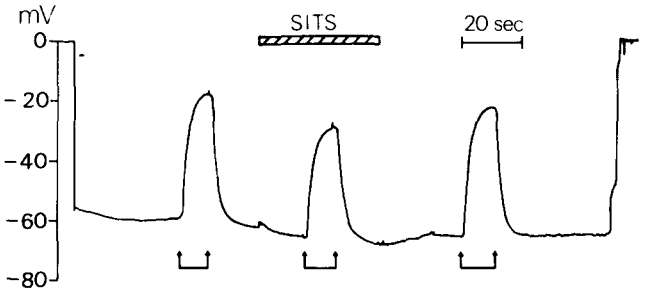

Fig. 2. Effect of chloride for bicarbonate substitution (the reversible switching from HCO_3 to HCO_3-free media is indicated each time by two consecutive arrows connected to each other) on membrane p.d., and the change elicited upon the basic response by the addition of SITS. The magnitude of the depolarization resulting from Cl/HCO_3 substitution is only moderately reduced by the addition of SITS.

two distinct mechanisms: (a) decrease of the transference number of potassium T_K and (b) diffusion potential Cl/HCO_3 (intracellular HCO_3 vs. extracellular Cl) arising from the removal of extracellular HCO_3 [19]. To get a rough estimate of the relative contribution of these two processes to the overall depolarization, single tubules were sequentially perfused with the following solutions: 1, control; 2, bicarbonate-free; 3, control; 4, addition of SITS (physiologic HCO_3); 5, bicarbonate-free plus SITS; 6, physiologic bicarbonate plus SITS; 7, control; 8, bicarbonate-free; 9, control (Fig. 2).

The depolarization elicited by bicarbonate-free media was always smaller when SITS was added to the solution ($\Delta V_{Cl,SITS}$) than when it was not (ΔV_{Cl}). The mean value of the $\Delta V_{Cl,SITS}/\Delta V_{Cl}$ ratio was 0.81 ± 0.04 mV, $n = 5$ ($P < 0.001$). Such observations suggest that the depolarization arising when chloride is substituted for bicarbonate in peritubular fluid proceeds from two mechanisms: (a) a diffusion potential Cl/HCO_3. Its contribution to the overall response is probably small. It may be estimated from the fractional decrease of the depolarizing response to bicarbonate-free media, when SITS is added into the solution. (b) The predominant effect of the removal of bicarbonate is the decrease of T_K, occurring probably via a decrease of P_K [19]. Similar studies in the rat have established that the Cl/HCO_3 depolarization mainly stems from a diffusion potential (Cl/HCO_3) because in these species the depolarizing response is almost completely abolished by the addition of SITS [20].

The average change in p.d. upon exposure of the tissue to bicarbonate-free solutions (SITS experiments not included) was 29.9 ± 1.8 mV, $n = 20$. The time course of change in p.d. was different in going from the bicarbonate-containing perfusate to bicarbonate-free perfusate ($t_{1/2} = 4.0 \pm 0.5$ s) than in going in the opposite direction ($t_{1/2}$ 2.6 ± 0.3 s). The comments already made with regard to the asymmetry in time course ('on' vs. 'off' responses) during exposure of the tissue to high-K media apply also to the data on bicarbonate-containing and bicarbonate-free solutions.

Conductance measurements. In four tubules membrane input conductance was continuously recorded in the control state and then upon reversible exposure to 0.5 mM SITS (Fig. 3). In each tubule the amplitude of the electrotonic potential during perfusion with solutions containing SITS (V_{SITS})

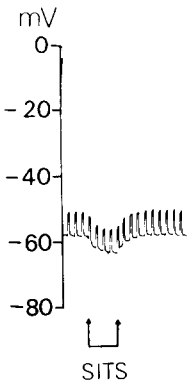

Fig. 3. Input conductance determination. Two microelectrodes were inserted into the cell layer of a single tubule and their distance was kept constant (200 μm in this experiment). One microelectrode was used to inject constant current pulses (20 nA in this experiment) and the second one for recording. The electrotonic potential (rectangular waves superimposed on membrane p.d.) result from current distribution along the 'cell cable', i.e. from the source of the recording electrode. The time of exposure to SITS is 20 s.

was expressed as a fraction of the corresponding electrotonic potential in the control state (V_{cont}). The mean value of V_{SITS}/V_{cont} was 0.99 ± 0.01, a figure not too different from 1.0 ($P > 0.2$). The failure to detect a decrease in conductance does not necessarily contraidct the data reported above. A small change in anionic permability yielding only 5 mV shift in p.d. may not produce detectable changes in input membrane conductance. In addition, one may not rule out a small increase of P_{Na} upon application of SITS, in *Necturus*, as in dog red blood cells [21]. This could offset the effects of reduced anionic permeabilities on membrane conductance, but oppose only in part their effect on membrane p.d.

Discussion

The effect of SITS on membrane p.d. was in great part reversible. This is not surprising since SITS is a disulfonic acid whose sulfonic groups combine reversibly with positive membrane charges of proper spacing and the isothiocyanate group reacts irreversibly with membrane amino groups in red cells [6,7]. The initial binding is the reversible one; the covalent binding, if it occurs, is a secondary and slower reaction [7,22]. Reversible effects of SITS have also been observed in other tissues [8,9]. It may be inferred that in the proximal tubule of *Necturus*, the essentially reversible effect of SITS is mediated through its sulfonic groups reversibly reacting with the membrane. It is not known to what extent this reaction occurs, i.e. what is the fractional saturation of the binding sites within 40 s of drug administration and therefore the degree of inhibition of membrane anionic permeabilities by SITS. However, no additional hyperpolarization was observed beyond 40 s in experiments in which the tissue was exposed to the drug for up to 3 min.

The observation that SITS hyperpolarizes the membrane when bicarbonate is present (in peritubular fluid) but fails to do so in bicarbonate-free media indicates that HCO_3 distribution across the peritubular membrane is not at equilibrium and also that SITS decreases P_{HCO_3}. Khuri et al. [4] reported

peritubular and intracellular bicarbonate concentrations to be almost equal, i.e. bicarbonate equilibrium potential, E_{HCO_3} would be less negative than E_m by nearly 70 mV. This statement implies that membrane permeability to bicarbonate, P_{HCO_3}, must be quite small. It is recalled that in the rat, current models on proximal bicarbonate reabsorption [23] make use of very high HCO_3 permeability at the peritubular membrane [24], a situation precluding large differences between E_{HCO_3} and E_m. Our present observations in *Necturus* do not allow a quantitative assessment of P_{HCO_3} or T_{HCO_3}. The small hyperpolarization elicited by addition of SITS is consistent either with a low value of P_{HCO_3} and large differences between E_{HCO_3} and E_m, as reported in *Necturus* by Khuri et al. [4], or values of E_{HCO_3} close to E_m, and with a high P_{HCO_3} as suggested in rat.

The failure of SITS to alter membrane p.d. in bicarbonate-free solutions (while it does so in the presence of bicarbonate) is at first glance surprising. It is consistent with, but does not prove, the concept that $E_{Cl} = E_m$. Such an assertion would be at variance with previous electrometric determinations suggesting that chloride distribution is not at equilibrium [2,3]. There are at least three ways to reconcile the results of others [2,3] and our present observations: (a) Postulate that the initial electrostatic binding of SITS, produced by the present (short-term) experiments, depressed selectively P_{HCO_3}, not P_{Cl}. This assertion appears unwarranted. (b) Assume that the physiologic value of P_{Cl} is negligibly small, making chloride contribution to membrane p.d. insigificant despite its non-equilibrium distribution. Thus, further reduction of P_{Cl} by SITS would not appreciably affect membrane p.d. (c) Assume that the presence of extracellular (or intracellular) bicarbonate is a prerequisite for the establishment of a measurable non-equilibrium chloride distribution, via some coupled HCO_3/Cl transport process or depression of P_{Cl} by the removal of peritubular bicarbonate.

There are no experimental observations presently available in favour of or against the first and third hypotheses stated above. The data reported in the literature as to the possible magnitude of P_{Cl} conflict, making it difficult to assess the validity of the second assumption. We have recently studied the effects of sixteen test-anions on membrane p.d. and found none of them to produce depolarization related to test-anion permeabilities smaller than P_{Cl} (in general, changes in p.d. were related to changes in T_K [13,25]). Spring and Kimura [3] have reported the intracellular chloride activity in proximal cells of *Necturus* to remain unaltered in spite of large changes in Cl electrochemical gradient across the peritubular membrane. From their mean values of E_m, $(Cl)_i$ and $(Cl)_0$ (−62 mV, 24.5 mM and 75 mM, respectively), one may estimate E_{Cl} at −28 mV and the driving force for Cl outflux across the peritubular membrane, $E_m - E_{Cl}$, at −34 mV. A somewhat lower figure was obtained by Khuri et al. [2]. Large differences in net electrochemical driving force are hardly consistent with high values of P_{Cl}, as is the insensitivity of $(Cl)_i$ to respond to the changes of the electrochemical Cl gradient [3] and also our SITS observations. Different conclusions have been reached by Boulpaep [15] who estimated the transference number of chloride (T_{Cl}) at the peritubular membrane to be at least 0.2 and the partial chloride conductance, g_{Cl}, to be 0.4. Such discrepancies are difficult to account for.

Our tentative conclusion is that in bicarbonate-free media chloride distribution is (or shifts) at electrochemical equilibrium. This is inferred from the failure of SITS to hyperpolarize the cell membrane when bicarbonate was removed, despite a concomitant decrease of T_K. The state of E_{Cl}, when peritubular bicarbonate is at physiologic concentrations cannot be assessed from the present data. If one assumes that chloride distribution is not at equilibrium [2,3] and $(Cl)_i$ is insensitive to variations of the chloride electrochemical gradient [3], then Boulpaep's figures of g_{Cl} and T_{Cl} [15] are probably overestimated. Conversely, high chloride permeability [15] would make rather unlikely the occurrence of a net electrochemical chloride gradient as high as 34 mV [3]. Further studies are required to elucidate this contradiction.

Acknowledgements

This study was supported by I.N.S.E.R.M. and D.G.R.S.T. grants.

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