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Inhibition of an Outwardly Rectifying Anion Channel by HEPES and Related Buffers

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Summary. The effect of pH buffers and related compounds on the conductance of an outwardly rectifying anion channel has been studied using the patch-clamp technique. Single-channel current-voltage relationships were determined in solutions buffered by trace amounts of bicarbonate and in solutions containing N-substituted taurines (HEPES, MES, BES, TES) and glycines (glycylglycine, bicine and tricine), Tris and *bis*-Tris at millimolar concentrations. HEPES (pK_a = 7.55) reduced the conductance of the channel when present on either side of the membrane. Significant inhibition was observed with 0.6 mM HEPES on the cytoplasmic side ($HEPES_i$) and this effect increased with [$HEPES_i$] so that conductance at the reversal potential was diminished ~25% with 10 mM $HEPES_i$ and ~70% at very high [$HEPES_i$]. $HEPES_i$ block was relieved by applying positive voltage but positive currents were not consistent with a Woodhull-type blocking scheme in that calculated dissociation constants and electrical distances depended on HEPES concentration. Results obtained by varying total $HEPES_i$ concentration at constant [$HEPES^-$] and vice versa suggest both the anionic and zwitterionic (protonated) forms of HEPES inhibit. Structure-activity studies with related compounds indicate the sulfonate group and heterocyclic aliphatic groups are both required for inhibition from the cytoplasmic side. TES (pK_a = 7.54), substituted glycine buffers (pK_a = 8.1–8.4) and *bis*-Tris (pK_a = 6.46) had no measurable effect on conductance and appear suitable for use with this channel.

Key Words: epithelia · chloride channel · cystic fibrosis · PANC-1 · TES

Introduction

Hydrogen ion activity has profound effects on biochemical processes and on transport through cell membranes. Protons can influence the conductance (e.g., Sigworth, 1977; Prod'hom, Pietrobon & Hess, 1987) and gating (Hanke & Miller, 1983) of ionic channels and also their selectivity (Vaughan, 1986). For these reasons the pH of solutions is carefully stabilized when studying channel properties. The buffer most often used for this purpose is HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), a zwitterionic amino acid that meets many of the criteria required of an ideal biological buffer (Good & Izawa, 1968). HEPES is relatively impermeant through cell membranes and has high buffering capacity in the physiological range (pH 6.8–8.2), its pK_a is not strongly affected by ionic strength, temperature or buffer concentration, and it does not form buffer-metal complexes readily or interfere with most chemical reactions. There are now many "Good" buffers extending the available pH range from 5.5 to 11.

In their original description of these compounds Good et al. (1966) pointed out that HEPES and its congeners can have undesirable side effects in some systems. HEPES has been shown to inhibit acetylcholine-induced currents in *Helix* neurons (Witte, Speckmann & Walden, 1985), antagonize cholinergic stimulation in the ampullar nerve of the semicircular canal (Norris & Guth, 1985), and competitively inhibit Na-independent binding of γ-aminobutyric acid (GABA) to its receptor (Tunnicliff & Smith, 1981). It also causes voltage-dependent flickering and multiple subconductance levels in a chloride channel from cultured *Drosophila* neurons (Yamamoto & Suzuki, 1987). Despite these precedents there have been few systematic studies of buffer effects at the single-channel level and HEPES continues to be used routinely in patch-clamp experiments.

Our laboratory has been studying an outwardly rectifying anion channel that has been implicated in epithelial chloride transport and cell volume regulation and closely resembles channels that function abnormally in cystic fibrosis (*see* Frizzell, 1987). During a recent study of HCO_3 permeability we noticed the channel had lower conductance when bathed by solutions containing HEPES (Tabcharani et al., 1989). It seemed important to characterize this effect more thoroughly considering the attention presently focused on this channel. The present study examines the effect of HEPES and related compounds on single-channel conductance. One

objective was to determine the voltage dependence and other characteristics of HEPES inhibition, since buffers could potentially be useful probes of chloride permeation if their action was fully understood. Another practical goal was to identify the pH buffers that are suitable when studying the biophysical properties of the channel.

A preliminary report of this work was presented at the Annual Meeting of the Society of General Physiologists, Woods Hole, Massachusetts, September 1989.

Materials and Methods

The PANC-1 cell line was obtained from American Type Culture Collection (Rockville, MD) and studied between passages 60 and 94. Cells were plated on glass coverslips at a density of 400,000/cm^2 and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml).

For patch recording, cells were transferred to a chamber containing simple NaCl solution (in mEq/liter): 154 Na, 154 Cl, 2 Ca, 10 HEPES, 1 ethylene glycol-*bis*(beta-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), pH 7.4; or a "normal bathing saline" (NBS) (in mEq/liter): 150 Cl, 144 Na, 4 K, 1 Mg, 2 Ca, 10 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 5 glucose, pH 7.4. In most experiments the pipette solution contained 150 mM NaCl, 2 mM $CaCl_2$ and 10 mM pH buffer (HEPES, tricine, etc.) and the pH was adjusted to 7.4 unless otherwise indicated. No effect of osmotic gradients on HEPES inhibition was detected during preliminary studies with 67 mOsm sucrose added to the bath, nevertheless sucrose was added to control solutions as an osmolyte when studying the effects of 25 and 50 mM HEPES. The chemicals used and their suppliers were: 1-piperidinepropionic acid, 1-(2-hydroxyethyl)piperazine, and ethanesulfonic acid from Aldrich Chemical, Milwaukee, WI; Tris(hydroxymethyl)methylamine (AnalaR grade) from BDH, Toronto, Ontario; methanesulfonic acid, Eastman Kodak, Rochester, NY; taurine (2-aminoethanesulfonic acid) and biological buffers, Sigma Chemical, St. Louis, MO. HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) was obtained from three independent sources (Sigma, Calbiochem-"Ultrol" grade, and BDH). The pH of each solution was adjusted using small quantities of sodium hydroxide or sulfuric acid. In control experiments sulfate had no effect on Cl permeation and its permeability was too low to alter the I/V relationship at the concentrations used in this study ($P_{SO4}/P_{Cl} = 0.19$). Some salines were buffered only by the trace levels of bicarbonate produced during equilibration with room air CO_2 (173 μM at pH 7.4); however, the pH of these solutions was tested frequently during experiments and remained stable within ± 0.15 pH units, a range that is too narrow to alter conductance significantly (*vide infra*). Moreover, the results obtained using weakly buffered solutions were identical to those obtained later with 10 mM concentrations of Tris, *bis*-Tris, and a variety of glycine-derived buffers. The final Cl activity of all solutions was checked using an ion-selective electrode. Experiments were carried out at 20°C with the exception of those designed to examine HEPES inhibition at 37 ± 1°C (Fig. 7).

Currents were recorded using inside-out patches (Hamill et al., 1981). Pipettes were pulled in two stages (PP-83, Narishige Scientific Instrument Lab., Tokyo) and had resistances of 4–6 MΩ when filled with 150 mM NaCl solution. The pipette contained a chlorided Ag wire; the bath was grounded through an agar bridge having the same ionic composition as the pipette solution. Currents were amplified using an Axopatch 1B (Axon Instruments, Burlingame, CA) or Yale Mk V patch clamp and recorded on video cassette tape by a pulse coded modulation-type recording adapter (DR384, Neurodata Instrument, NY). Single-channel records were low-pass filtered using an 8-pole Bessel filter (902LPF, Frequency Devices, Haverhill, MA) set at 600 Hz (final cutoff frequency 514 Hz) and sampled at 0.5 or 1.0-msec intervals.

Data were analyzed using a laboratory microcomputer system (Indec Systems, Sunnyvale, CA) as described previously (Tabcharani et al., 1989; Hanrahan & Tabcharani, 1989). Briefly, current-voltage (I/V) relationships were calculated using a semi-automated procedure that involved computing amplitude histograms for short segments of record. These were displayed on a split screen next to the raw data so that peaks could be verified using cursors. At least 10 open events were measured at each steady-state potential, averaged, and entered into an I/V curve that was displayed at the end of the run. Slope conductance was determined by linear regression over the voltage ranges specified in Results.

Significant difference was determined at the 95% confidence level using paired or unpaired Student's t tests.

Results

Outwardly rectifying anion channels were observed in approximately 75% of patches that had been excised and held at +60 mV for several minutes at 20°C. The frequency of observing channels was not increased when the temperature was elevated to 37°C; however, channels usually became active spontaneously after excision, making large voltages unnecessary (Low, Tabcharani & Hanrahan, 1990). Patches that contained anion channels usually had at least two and sometimes contained more than eight channels. The anion channel was distinctive and easily recognized by its outward rectification and voltage dependence.

Figure 1*a* shows records obtained at +60 and −60 mV when the channel was bathed on both sides by nominally buffer-free (except for trace bicarbonate) 150 mM NaCl at 20°C. Figure 1*b* shows the same channel after replacing the bath solution with one containing 10 mM HEPES. $HEPES_i$ clearly reduced the current amplitude, and inhibition was strongest at negative potentials. Figure 1*c* compares the mean current-voltage relationship obtained with 10 mM HEPES (pH 7.4, 20°C) on the intracellular side with the control relationship obtained in symmetrical, nominally buffer-free solution. Although currents were reduced at large positive potentials the slope conductance above 50 mV was not changed significantly (76 *vs.* 77 pS at 60 mV; $P > 0.1$) because the rectification was steeper between

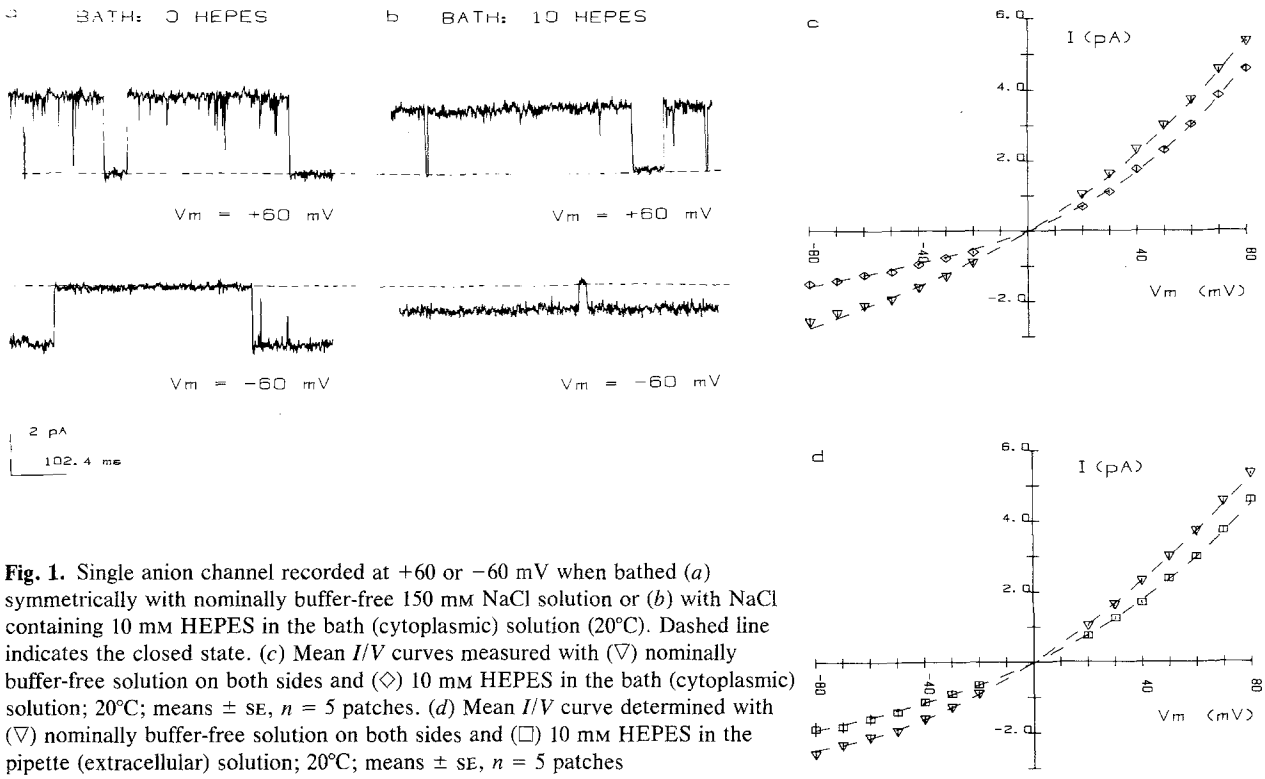

Fig. 1. Single anion channel recorded at +60 or −60 mV when bathed (*a*) symmetrically with nominally buffer-free 150 mM NaCl solution or (*b*) with NaCl containing 10 mM HEPES in the bath (cytoplasmic) solution (20°C). Dashed line indicates the closed state. (*c*) Mean *I/V* curves measured with (▽) nominally buffer-free solution on both sides and (◇) 10 mM HEPES in the bath (cytoplasmic) solution; 20°C; means ± SE, $n = 5$ patches. (*d*) Mean *I/V* curve determined with (▽) nominally buffer-free solution on both sides and (□) 10 mM HEPES in the pipette (extracellular) solution; 20°C; means ± SE, $n = 5$ patches

20 and 40 mV with $HEPES_i$. The slope conductance at negative potentials (between −70 and −50 mV) decreased from 17.5 ± 2.5 pS to 12.5 ± 2.1 pS with 10 mM $HEPES_i$ and from 46.3 ± 2.1 pS to 31.2 ± 0.45 pS at the reversal potential. The latter value is similar to that reported previously when the channel is bathed symmetrically with 10 mM HEPES (30.3 ± 0.7 pS at 0 mV; 20°C; Tabcharani et al., 1989), suggesting the HEPES effect is strongest from the cytoplasmic side. This is confirmed in Fig. 1*d*, which shows the inhibition of conductance caused by extracellular HEPES (10 mM; pH 7.4, 20°C). Inhibition by extracellular HEPES was slightly weaker than with $HEPES_i$, particularly at negative potentials. The mean conductance was 35.7 pS with 10 mM $HEPES_o$ at 0 mV and 20°C.

Figure 2*a* shows the effects of raising [$HEPES_i$] on the mean current-voltage relationship. Single-channel currents were reduced at all potentials and rectification became more pronounced as [$HEPES_i$] was increased stepwise from 0 to 50 mM or decreased stepwise from 50 mM to 0 (pH 7.4; 20°C). The effect of HEPES was completely reversible. Figure 2*b* shows relative block as a function of voltage, normalized to the currents measured in HEPES-free solution (I_b/I_o), where I_b and I_o are the single-channel currents measured with and without HEPES, respectively. Increasing [$HEPES_i$] from 1 to 50 mM progressively reduced I_b/I_o at all potentials. Surprisingly, $HEPES_i$ inhibition was independent of voltage at negative potentials but was progressively relieved by clamping the membrane potential to increasingly positive voltages. The data obtained at each HEPES concentration could be fitted by two straight lines that intersect near 0 mV. As a first approach we assumed the inhibition at positive potentials was due to $HEPES^-$ interacting with a site within the membrane field so that binding and unbinding rates would depend on voltage (Woodhull, 1973). The dissociation constant at 0 mV [$K_d(0)$] and the electrical distance of the site within the field (δ) were then estimated using

$$I_o/I_b = 1 + \frac{[\text{HEPES}]}{K_d(0)} \exp(z\delta V_m F/RT)$$

where I_b and I_o are single-channel currents measured in the absence and presence of $HEPES_i$, respectively, and z, F, R and T have their usual meanings. Figure 2*c* shows the relationship between V_m and $\ln[(I_o/I_b) - 1]$ for the data obtained with 25 mM total [$HEPES_i$]. A straight line fits these positive currents reasonably well ($r^2 = 0.99$), yielding $K_d(0)$ = 6.47 mM $HEPES^-$ and electrical distance = 44.7% of the membrane field from the inside. Data obtained with 10 mM $HEPES_i$ gave similar estimates of 6.9 mM and 48.5% for these parameters, respectively; however, very different estimates

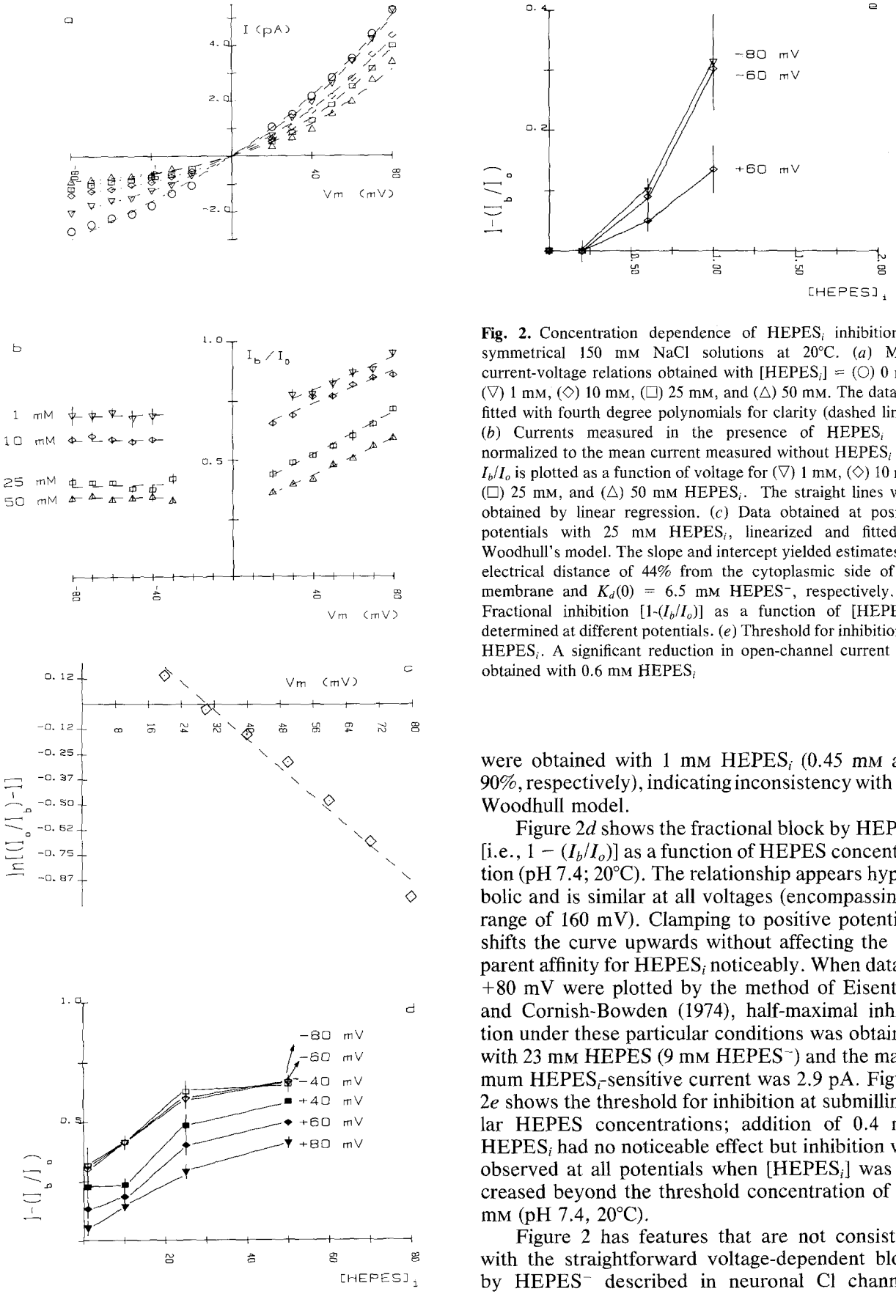

Fig. 2. Concentration dependence of $HEPES_i$ inhibition in symmetrical 150 mM NaCl solutions at 20°C. (*a*) Mean current-voltage relations obtained with [$HEPES_i$] = (○) 0 mM, (▽) 1 mM, (◇) 10 mM, (□) 25 mM, and (△) 50 mM. The data are fitted with fourth degree polynomials for clarity (dashed lines). (*b*) Currents measured in the presence of $HEPES_i$ (I_b), normalized to the mean current measured without $HEPES_i$ (I_o). I_b/I_o is plotted as a function of voltage for (▽) 1 mM, (◇) 10 mM, (□) 25 mM, and (△) 50 mM $HEPES_i$. The straight lines were obtained by linear regression. (*c*) Data obtained at positive potentials with 25 mM $HEPES_i$, linearized and fitted to Woodhull's model. The slope and intercept yielded estimates for electrical distance of 44% from the cytoplasmic side of the membrane and $K_d(0)$ = 6.5 mM $HEPES^-$, respectively. (*d*) Fractional inhibition [$1-(I_b/I_o)$] as a function of [$HEPES_i$], determined at different potentials. (*e*) Threshold for inhibition by $HEPES_i$. A significant reduction in open-channel current was obtained with 0.6 mM $HEPES_i$

were obtained with 1 mM $HEPES_i$ (0.45 mM and 90%, respectively), indicating inconsistency with the Woodhull model.

Figure 2*d* shows the fractional block by HEPES [i.e., $1 - (I_b/I_o)$] as a function of HEPES concentration (pH 7.4; 20°C). The relationship appears hyperbolic and is similar at all voltages (encompassing a range of 160 mV). Clamping to positive potentials shifts the curve upwards without affecting the apparent affinity for $HEPES_i$ noticeably. When data at +80 mV were plotted by the method of Eisenthal and Cornish-Bowden (1974), half-maximal inhibition under these particular conditions was obtained with 23 mM HEPES (9 mM $HEPES^-$) and the maximum $HEPES_i$-sensitive current was 2.9 pA. Figure 2*e* shows the threshold for inhibition at submillimolar HEPES concentrations; addition of 0.4 mM $HEPES_i$ had no noticeable effect but inhibition was observed at all potentials when [$HEPES_i$] was increased beyond the threshold concentration of 0.6 mM (pH 7.4, 20°C).

Figure 2 has features that are not consistent with the straightforward voltage-dependent block by $HEPES^-$ described in neuronal Cl channels

(Yamamoto & Suzuki, 1987). The ratio I_b/I_o is independent of voltage at $V_m < 0$ mV (Fig. 2*b*) although this ratio should continue to decline as the membrane potential is clamped to increasingly negative voltages according to the Woodhull model. One would expect, for example, that inhibition by 4.44 mM $HEPES^-$ (10 mM HEPES at pH 7.4) would approach that caused by 11.1 mM HEPES (25 mM $HEPES_i$ at pH 7.4) when the voltage is made sufficiently negative, but this was not observed. The results imply that inhibition is not mediated exclusively by the anionic (unprotonated) form of HEPES.

To determine if zwitterionic HEPES is also inhibitory, channels were bathed with solutions containing different (total) concentrations of $HEPES_i$ at a fixed [$HEPES_i^-$]; 20°C. Figure 3*a* shows *I/V* relationships determined at pH 8.25 without HEPES on either side of the membrane and in the presence of 13 mM $HEPES_i$ (calculated [$HEPES_i^-$] = 11.1 mM). Figure 3*b* shows the *I/V* relationships obtained before and after adding 67 mM $HEPES_i$ at pH 6.8 (again [$HEPES_i^-$] = 11.1 mM). As expected, *I/V*s obtained in the absence of $HEPES_i$ at pH 6.8 and 8.25 revealed a slight (buffer-independent) inhibition of conductance by alkaline pH; conductance at 0 mV decreased from 44.6 to 37.8 pS when pH was elevated from 6.8 to 8.25 (Fig. 3*a,b*). More importantly, $HEPES_i$ inhibited under both conditions but was clearly more potent at pH 6.8. This is shown in Fig. 3*c* by replotting I_b/I_o (the current measured with $HEPES_i$, normalized to the control current measured at the same pH without $HEPES_i$). The fact that inhibition was stronger at all potentials with 67 mM $HEPES_i$ (pH 6.8) than with 13 mM $HEPES_i$ (pH 8.25) even though the calculated concentration of $HEPES^-$ was identical suggests the protonated (zwitterionic) form of HEPES is inhibitory. Other interpretations are possible, however, because [$HEPES_i$] and pH were both varied, and pH might have affected the channel independently of buffer dissociation.

To exclude this possibility the effect of 25 mM $HEPES_i$ was compared at pH 8.2 (~85% of the HEPES in the anionic form; Fig. 4*a*) and at pH 6.5 (~87% in the zwitterionic form; Fig. 4*b*). Control currents measured at pH_i = 8.25 were slightly reduced compared to those measured at pH 6.5 as shown above but $HEPES_i$ inhibition and its relief by positive voltage was similar at both pHs despite their very different HEPES : $HEPES^-$ ratios. These data, when combined with the similar effects of MES and MOPS described below (pK_a = 6.1 and 7.2, respectively), suggest that both forms of HEPES are effective and that enhanced inhibition observed in Fig. 3 at pH 6.8 was due to inhibition by zwitterionic $HEPES_i$ rather than low pH.

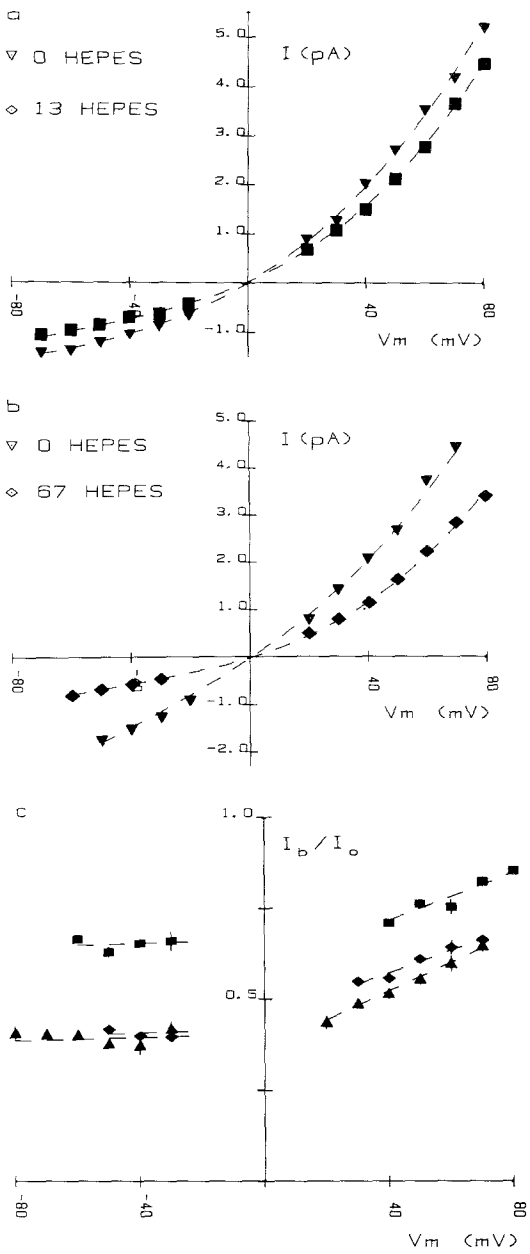

Fig. 3. Effect of varying (total) $HEPES_i$ concentration while maintaining constant $HEPES^-$ concentration at 20°C. (*a*) Mean *I/V* relationships determined at pH 8.25 with (▼) 0 mM and (■) 13 mM HEPES bathing the cytoplasmic side. When total [$HEPES_i$] is 13 mM and pH = 8.25, calculated [$HEPES^-$] = 11.06 mM. (*b*) *I/V* relationships determined at pH 6.8 with (▼) 0 mM and (◆) 67 mM $HEPES_i$, calculated [$HEPES^-$] = 11.06 mM. (*c*) Currents measured with (■) 13 mM and (◆) 67 mM $HEPES_i$ at pH 8.25 and 6.8, respectively. Data were normalized to control currents measured without HEPES at the same pH. For comparison, (▲) shows the relation for 25 mM $HEPES_i$ at pH 7.4

Do $HEPES_i$ and chloride compete for a common site on the channel? Figure 5*a* shows the mean *I/V* curves obtained with symmetrical 60 mM NaCl and different concentrations of $HEPES_i$. Currents measured in 60 mM NaCl solutions were approxi-

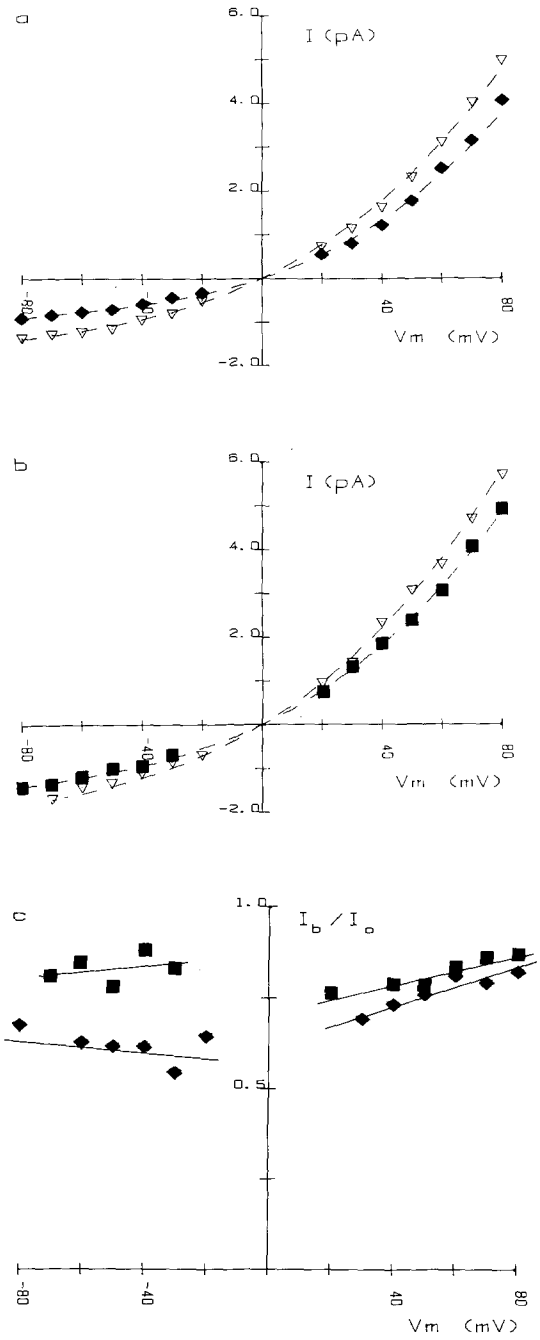

Fig. 4. Effect of varying [HEPES$^-$] at constant (total) HEPES$_i$ concentration at 20°C. (*a*) *I/V* relationships determined at pH 8.25 with (▽) 0 mM and with (◆) 25 mM HEPES bathing the cytoplasmic side. The concentration of HEPES$^-$ is calculated to be 21.3 mM at this pH. (*b*) *I/V* relationships determined at pH 6.5 with (▽) 0 mM and (■) 25 mM HEPES$_i$. The calculated [HEPES$^-$] is 3.25 mM under these conditions. (*c*) Inhibition is stronger at all potentials when pH = 8.25 compared to pH 6.5, but its voltage dependence at positive potentials is not altered

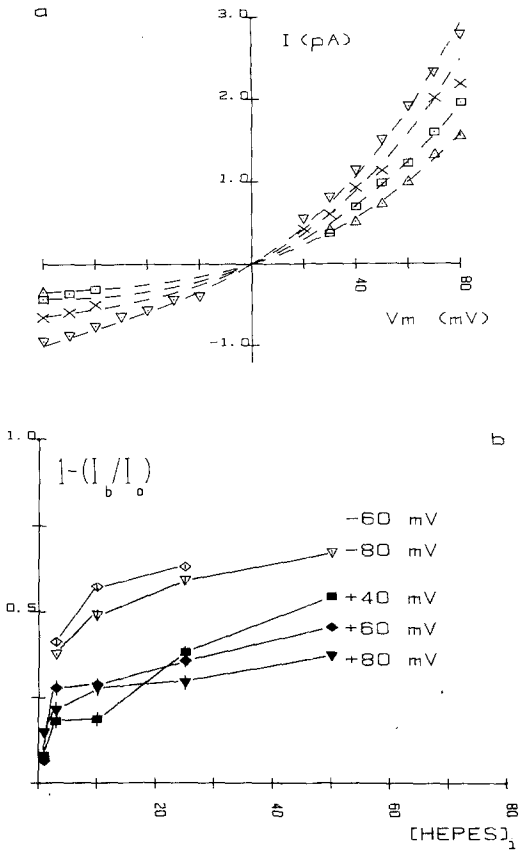

Fig. 5. Concentration dependence of HEPES$_i$ inhibition in symmetrical 60 mM NaCl at 20°C. (*a*) Mean *I/V* curves obtained in the absence of HEPES (▽) and with (×) 3 mM, (□) 25 mM and (△) 50 mM HEPES$_i$; means ± SE, n = 3–5. The data have been fitted with fourth degree polynomials for clarity (dashed lines). (*b*) Fractional inhibition in symmetrical 60 mM NaCl solution at different potentials, plotted as a function [HEPES$_i$]

mately half those in 150 mM NaCl solutions, and HEPES$_i$ again reduced the current amplitudes and increased rectification. However, half-maximal inhibition was produced by 8 mM HEPES$_i$, approximately one-third the concentration required in 150 mM NaCl solutions. This suggests competition between chloride and HEPES$_i$ although it does not rule out nonspecific (e.g., lyotropic) effects on HEPES$_i$ binding. Voltage-dependent relief from HEPES$_i$ inhibition in 60 mM NaCl solution was almost identical to that seen with 150 mM NaCl.

The potency of HEPES$_i$ was influenced by anion permeation from the "*trans*" side. Figure 6 compares block by HEPES$_i$ when the channel was bathed symmetrically with 150 mM NaCl solution and when the pipette solution contained 270 mM NaCl (pH 7.4; 20°C). Elevating pipette [Cl] 1.8-fold reduced inhibition by bath HEPES (i.e., increased I_b/I_o) at all potentials without affecting the slope at $V_m > 0$ mV, and the same result was obtained when iodide was substituted for Cl in the pipette solution (Fig. 6; P_I/P_{Cl} ~1.8; Halm et al., 1988; our *unpublished observations*). However, there was no correlation between the level of block and current amplitude. In other words, currents measured at +70 mV in symmetrical 150 mM NaCl solution were much larger than those measured at +20 mV with high

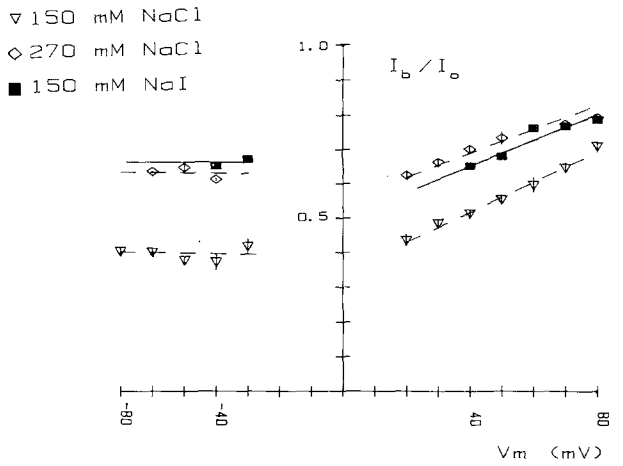

Fig. 6. Effect of high extracellular [Cl] and iodide substitution on the inhibition by $HEPES_i$. Currents measured in the presence of 25 mM $HEPES_i$ were normalized to those measured without HEPES; 20°C; means ± SE. The bath contained 150 mM NaCl under all conditions; the pipette contained (▽) 150 mM NaCl, (◇) 270 mM NaCl or (■) 150 mM NaI. I_b/I_o was increased (i.e., inhibition by $HEPES_i$ was relieved) at all potentials when pipette [Cl] was elevated 1.8-fold or replaced with iodide (P_I/P_{Cl} = 1.8)

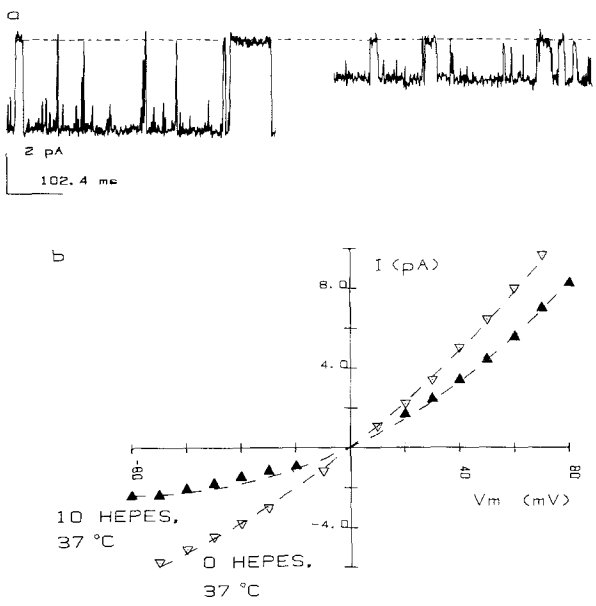

Fig. 7. (*a*) Current recordings obtained at V_m = −60 mV and 37°C in the absence of $HEPES_i$ (left) and with 10 mM $HEPES_i$ (right). (*b*) *I/V* relationships for conditions used in *a* above. (▽) 0 mM $HEPES_i$, (▲) 10 mM $HEPES_i$; data are representative of five experiments

pipette [Cl] or with iodide in the pipette, even though the ratio I_b/I_o was identical under these conditions. This argues against a simple "knock off" mechanism as the basis of relief from $HEPES_i$ block, but the relief might still be explained by competition between $HEPES_i$ and permeating anions at the cytoplasmic end of the channel.

Inhibition by $HEPES_i$ was accentuated when the temperature was increased from 20 to 37°C (Fig. 7). The *I/V* relationship was surprisingly linear at 37°C in the absence of HEPES, although outward rectification at this temperature was markedly increased by 10 mM $HEPES_i$. At −60 mV, HEPES (10 mM) reduced the conductance by 29% at 20°C and 55% at 37°C. Most of the outward rectification observed at 37°C is an artifact caused by the buffer.

Effects of HEPES-Related Compounds

A variety of buffers and HEPES-related compounds were studied to gain more insight into interactions between pH buffers and the channel; most of their structures are shown in Fig. 8. We examined glycine buffers, Tris and *bis*-Tris first because they are unrelated to HEPES and might be useful in subsequent control solutions. Figure 9*a* shows examples of current amplitude histograms obtained at −50 mV (left) and +50 mV (right) in the (*i*) absence of buffer, and with (*ii*) 10 mM tricine, (*iii*) 10 mM glycylglycine, and (*iv*) 10 mM *bis*-Tris on the cytoplasmic side. It is apparent from the areas under the peaks and distances between them that these three buffers had little effect on open probability or current amplitude. Tricine was chosen for more detailed study because it had no apparent effect on conductance and its pK_a is closer to physiological pH than other glycine-derived buffers. Figure 9*b* compares records obtained at −50 mV (left) and +50 mV (right) in the absence (upper traces) and presence (lower traces) of tricine and also illustrates amplitude histograms determined in the absence

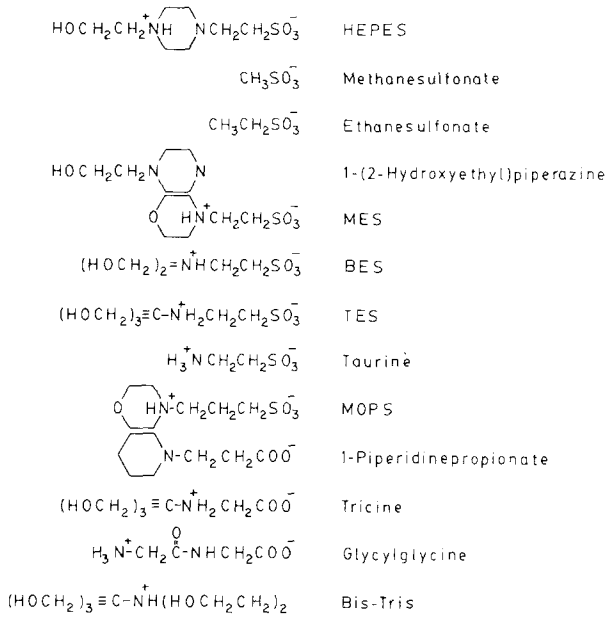

Fig. 8. Chemical structures of buffers and related compounds used in this paper

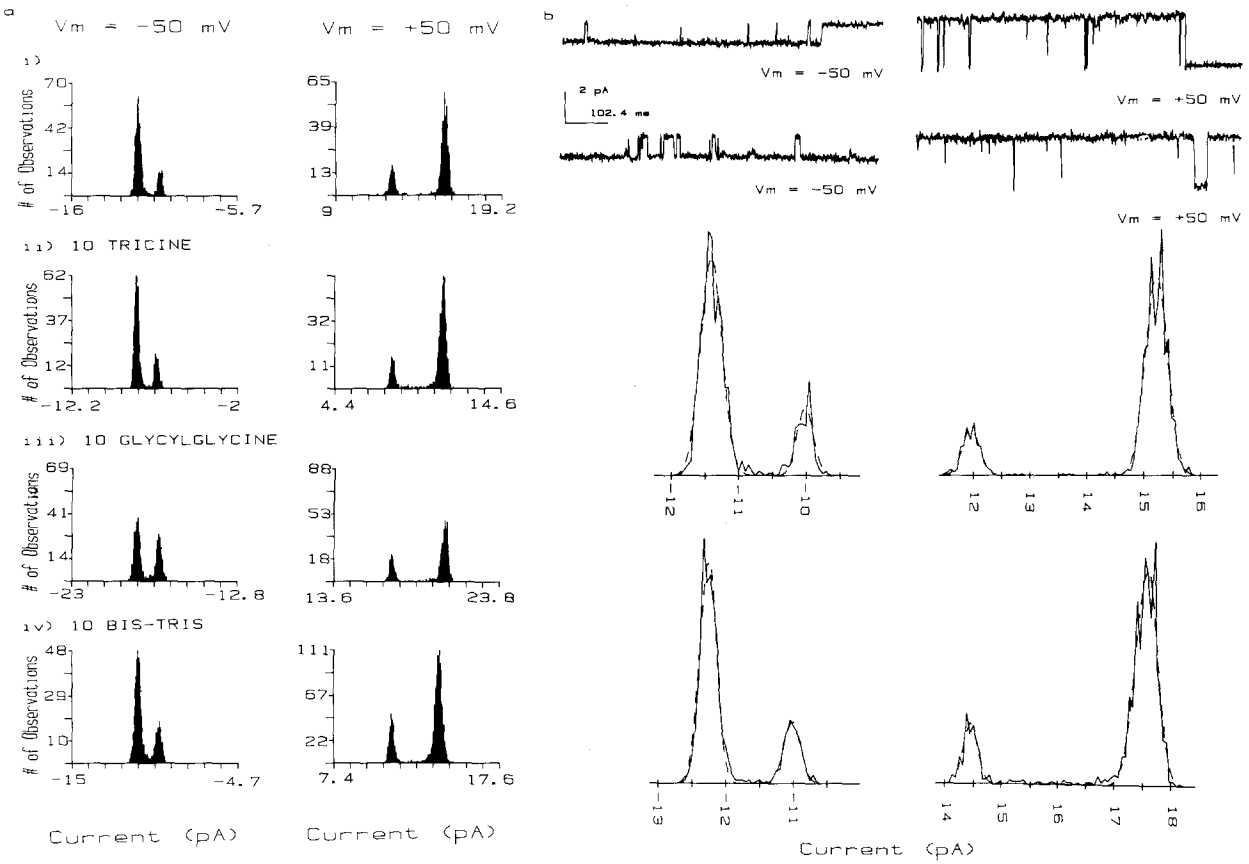

Fig. 9. (*a*) Chemical amplitude histograms obtained at −50 mV (left) and +50 mV (right) when the cytoplasmic side of the patch was bathed with (*i*) nominally buffer-free solution, (*ii*) 10 mM tricine, (*iii*) 10 mM glycylglycine and (*iv*) 10 mM *bis*-Tris; 20°C. (*b*) Examples of records and amplitude histograms (with Gaussian fits) at −50 mV (left) and +50 mV (right) in the absence of buffer (upper traces and histograms) and with 10 mM tricine (lower traces and histograms). There was no effect of tricine on conductance as determined from the distance between fitted means. Representatives of three experiments except tricine ($n = 6$)

(upper histograms) and presence of tricine (lower histograms). The open-channel current amplitude was estimated from the separation between mean open and closed current levels after fitting the peaks with Gaussian curves (e.g., Fig. 9*a*,*b*). Tricine, glycylglycine, and bicine did not affect channel conductance significantly nor did *bis*-Tris ($pK_a = 6.46$) or Tris ($pK_a = 8.3$), although the latter caused some channels to flicker. The mean single-channel conductance between −40 and −60 mV was 25 ± 2.08 pS in nominally buffer-free solution and 23.5 ± 2.24 pS (NS $P > 0.2$) with 10 mM $tricine_i$. These values are not different from each other but are higher than those obtained with HEPES-buffered solutions (14.4 ± 1.02; sign., $P < 0.05$). Once it became clear that tricine had no effect on channel conductance it was used in control solutions when testing the effects of aminosulfonates.

TES was the only HEPES-related buffer studied that had no effect on conductance when present on either side of the membrane. Figure 10*a* shows traces from a single channel exposed successively to cytoplasmic (*i*) 10 mM tricine, (*ii*) 10 mM TES and (*iii*) 10 mM HEPES. The kinetics and current amplitudes were qualitatively similar with tricine and TES, and this was not due to a lack of sensitivity of this particular channel since subsequent exposure to 10 mM HEPES caused inhibition. Figure 10*b* shows that the *I*/*V* curves obtained in the nominal absence of buffer on the cytoplasmic side and with 10 mM tricine or 10 mM TES could be superimposed. Identical *I*/*V* relations were obtained with symmetrical TES-buffered solutions. By contrast, Fig. 10*c* shows *I*/*V*s obtained with 10 mM HEPES, MOPS or MES in the bath. These buffers reduced channel conductance to 53.3% (HEPES), 55.5% (MOPS) and 62.5% (MES) of controls (tricine) when measured at ~ −70 mV. The fact that these different buffers caused similar inhibition strengthens the notion that both protonated and unprotonated buffers are inhibitory because their pK_as (and therefore anion-to-zwitterion ratios at pH 7.4) are

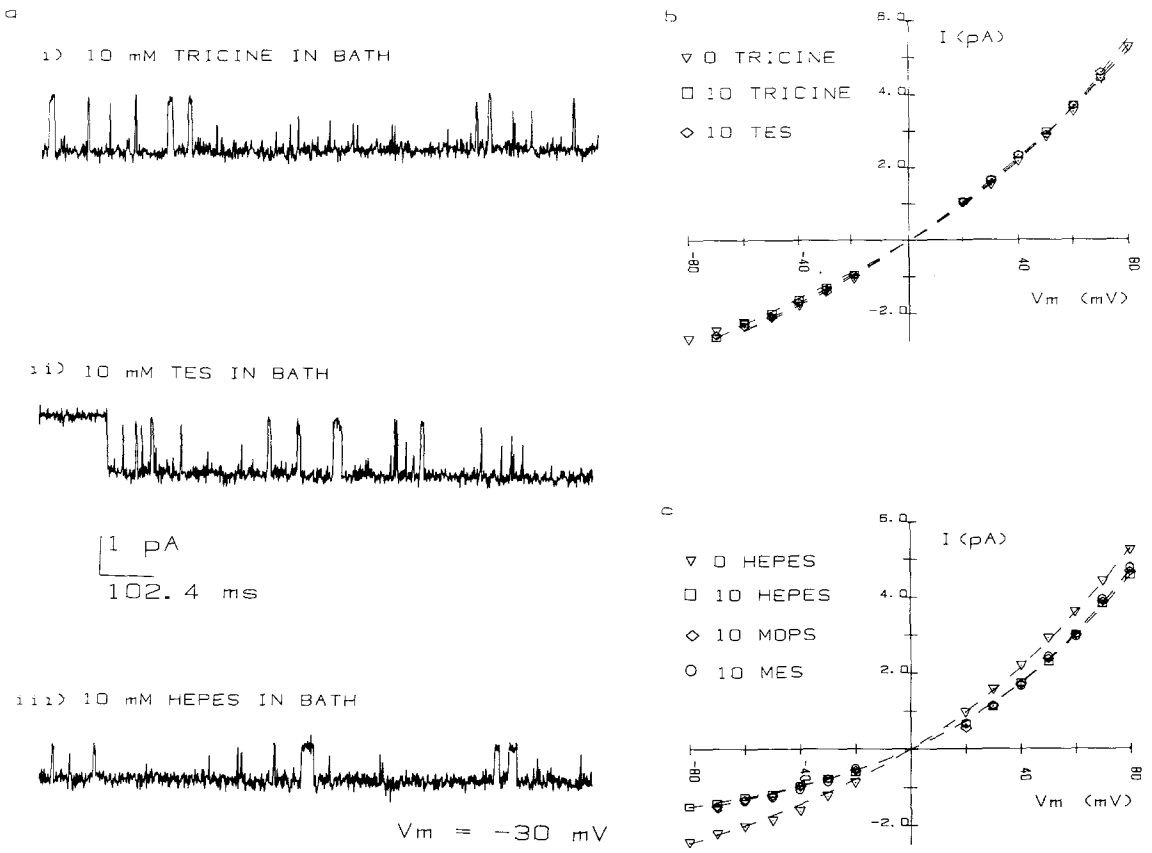

Fig. 10. (*a*) Single-channel records during successive exposure to 150 mM NaCl solutions containing (*i*) 10 mM tricine, (*b*) 10 mM TES and (*c*) 10 mM HEPES at 20°C. (*b*) Representative *I/V* relationships obtained at 20°C with 10 mM tricine and TES. (*c*) *I/V* curves obtained at 20°C with three different inhibitory buffers; HEPES, MOPS and MES; means of 4–5 patches, standard errors are smaller than the symbols

very different. Methanesulfonate (10 mM), ethanesulfonate (10 mM), and 1-(2-hydroxyethyl)piperazine (10 mM), which are constituents of HEPES, had no effect from the cytoplasmic side (Table 1). Moreover, taurine, which is related to HEPES and is present at high intracellular levels in some tissues, also had no effect (Table 1).

The data suggest that any taurine-related buffer containing a heterocyclic aliphatic group will probably inhibit the outward rectifier; however, the absence of such a group does not ensure it will be innocuous. Each buffer needs to be tested from both sides; we found that BES (10 mM) had little effect from the cytoplasmic side (Table 1) but was as inhibitory as HEPES in the extracellular solution. Table 2 summarizes the results obtained with buffers in the extracellular solution: Tricine_o (10 mM) and TES_o (10 mM) had no effect on the conductance calculated at 0 mV. Inhibition by HEPES at the reversal potential was similar from either side of the membrane whereas inhibition by BES was strongest from the outside. Buffers presumably interact with intra- and extracellular sites having slightly different properties.

Discussion

The outwardly rectifying anion channel is widely distributed among epithelial and nonepithelial cells and has received increasing attention since its modulation by cAMP was reported to be defective in cystic fibrosis (Schoumacher et al., 1987; Li et al., 1988). The channel has been characterized in some detail but patch-clamp and bilayer experiments have usually been carried out in HEPES-buffered solutions. In this paper we have shown that HEPES and certain other N-substituted taurines reduce the conductance of the channel by approximately half at physiological potentials when present on the cytoplasmic side of the membrane. The inhibition increases with temperature and accounts for most of the outward rectification observed at 37°C. We have only characterized effects on conductance, but it is

Table 1. Effect of HEPES and related compounds on conductance of the outwardly rectifying anion channel when tested from the cytoplasmic side

HEPES (5)	75.1 ± 2.6%[b]
Methanesulfonate (2)	95.3 ± 2.4%
Ethanesulfonate (1)	103.4%
1-(2-Hydroxyethyl)piperazine (2)	97.6 ± 2.6%
MES (4)	72.7 ± 3.7%[b]
BES (2)	94.0 ± 1.0%
TES (3)	100.4 ± 0.37%
Taurine (1)	106.7%
MOPS (3)	74.9 ± 4.5%[b]
1-Piperidinepropionate (3)	98.1 ± 1.4%
Tricine (6)	102 ± 5.5%
Bicine (2)	94.9 ± 4.1%
Glycylglycine (5)	112.4 ± 2.0%
Bis-Tris (3)	99.41 ± 0.35%

[a] Conductance at the reversal potential relative to that measured in nominally buffer-free solution at 20°C. (*n*).
[b] Difference significant at $P < 0.05$.

Table 2. Effect of extracellular HEPES and other buffers on the conductance of the outwardly rectifying anion channel

HEPES (4)	77.3 ± 3.6%[b]
BES (4)	81.9 ± 2.8%[b]
TES (3)	101.6 ± 2.8%
Tricine (5)	95.2 ± 3.3%

[a] Conductance at the reversal potential relative to that measured in nominally buffer-free solution at 20°C. (*n*).
[b] Difference significant at $P < 0.05$.

important to note that buffers could have other actions. For example fast block by HEPES could influence the apparent kinetics of the channel by prolonging bursts of openings, and HEPES might influence the pharmacological properties of the channel by protecting it from transport inhibitors and site-specific reagents.

The voltage dependence of HEPES inhibition is unique and cannot be attributed to block by anionic $HEPES^-$ or to simple dual block by the protonated and unprotonated forms of the buffer. Moreover, HEPES and Cl apparently compete, and this could influence voltage-dependent and -independent components of the $HEPES_i$ inhibition. It is difficult to predict what effects a transmembrane field would have on interactions between zwitterionic HEPES and the channel. Regardless, it is clear that inhibition is partially relieved by positive voltage and thus contributes to outward rectification in the I/V relationship. The threshold for HEPES inhibition was 0.6 mM; at very high HEPES concentrations ~70% inhibition was achieved. Nearly all experiments in this study were carried out at 20°C but results in Fig. 7 indicate relative inhibition is more pronounced at 37°C. The efficacy of $HEPES_i$ as a channel inhibitor is comparable to some commonly used K and Na channel blockers. For example, the affinity of $HEPES_i$ for the outward rectifier is stronger than that of TEA_i for Ca-activated maxi-K channels (Vergara & Latorre, 1983; Blatz & Magleby, 1984).

Is Inhibition Caused by the pH Buffer Itself or by a Contaminant?

If inhibition is caused by some impurity, the contaminant must be present at identical concentrations in HEPES from three independent sources (Sigma, Calbiochem, and BDH), in regular and ultrapure grades of HEPES (>99%; Sigma, Calbiochem), in HEPES prepared as the free acid and sodium salt, and in preparations of other buffers such as MES and MOPS. The putative contaminant is unlikely to be a reactant used during buffer synthesis because some inhibitory buffers are prepared from different starting compounds; for example, HEPES is prepared from N-(2-hydroxylethyl)piperazine and sodium chloroethanesulfonate, whereas MOPS is synthesized from morpholine and propane sultone. Also, we directly tested some reactants (e.g., N-(2-hydoxyethyl)piperazine and ethanesulfonate) and they had no effect. The procedures used to prepare MES and BES are almost identical and should have similar impurities yet MES inhibited from the cytoplasmic side while BES did not. We cannot exclude the possibility that a contaminant is responsible for the effects described in this paper; however, all the indirect evidence suggests channel inhibition is caused by the buffers.

Mechanism of Inhibition

The effect of HEPES on current amplitude is reminiscent of blockers such as TEA that interact with channels at rates that are too rapid to be resolved; however, several observations are inconsistent with the simple Woodhull-type blocking scheme normally used for such blockers. First, inhibition at negative potentials was independent of voltage; in other words, the relationship between I_b/I_o and membrane potential had zero slope at $V_m < 0$ mV but increased abruptly at $V_m > 0$ mV. This behavior is not consistent with a simple dependence of blocking and unblocking rates on voltage. Second, the voltage-dependent portion of the I_b/I_o relationship yields different estimates for electrical distance and $K_d(0)$ when fitted with a Woodhull-type model at different $HEPES_i$ concentrations; the parameters derived from this model are not expected to be concentration dependent. Third, the results in Figs. 3 and 4 suggest that both protonated (no net charge)

and unprotonated (negatively charged) forms of $HEPES_i$ are inhibitory. Increasing the membrane potential from −80 to 80 mV causes a downward shift in the concentration-inhibition relationship without altering its shape, suggesting voltage does not strongly influence HEPES-channel interactions over the concentration range 1–50 mM $HEPES_i$ (Figs. 2*d* and 5*b*). Also, the same voltage-dependent behavior was observed for all ratios of protonated : unprotonated $HEPES_i$.

$HEPES_i$ may cause a rapid, partial block of the outward rectifier so that conductance "substates" are too brief to be resolved. This hypothesis is attractive because it is consistent with HEPES-induced substates reported in neuronal Cl channels at low temperature (Yamamoto & Suzuki, 1987), and partial block could explain why HEPES inhibition is incomplete at the highest concentrations used in the present study (note that ~30% of the current at 20°C was HEPES insensitive). In neurons, HEPES inhibition was assumed to result from block by anionic $HEPES^-$ (fully closed) and the voltage dependence was analyzed accordingly; however, the probability of low-conductance substates was also increased by HEPES in that study and substates were omitted from the analysis of voltage dependence (Yamamoto & Suzuki, 1987). One possibility is that HEPES-induced substates reflect partial block by the zwitterion. Partial block by zwitterions might also contribute to the voltage-independent component of $HEPES_i$ inhibition observed in the present study.

Elevating pipette [Cl] or replacing chloride in the pipette solution with iodide relieves $HEPES_i$ inhibition, but the magnitude of relief does not correlate with membrane potential or current flow, arguing against a simple knock-off effect. Increased HEPES-anion competition at the cytoplasmic end of the channel might explain relief from inhibition by $HEPES_i$ if enhanced permeation from the *trans* side increased the occupancy of a shared site by anions. The data obtained with 60 mM Cl suggests there is indeed some Cl-$HEPES_i$ competition. Alternatively, relief of $HEPES_i$ block by extracellular anions maybe related to "*trans*"-stimulation in which low levels of Cl on one side of the membrane enhance anion permeation from the opposite side (Halm & Frizzell, 1988; Tabcharani et al., 1989).

Structural Features of pH Buffers Relevant to Channel Block

Several HEPES-related compounds were studied in an attempt to learn more about interactions between buffers and the channel. Alkylsulfonates were tested first because the negatively charged sulfonate seemed most likely to be involved in binding to the channel (Fig. 8). Surprisingly, methanesulfonate and ethanesulfonate (10 mM) had no effect nor did 1-(2-hydroxyethyl)piperazine, implicating both parts of the HEPES molecule in channel interactions. MES_i (10 mM), an N-substituted taurine with a different aliphatic heterocyclic group had the same effect as $HEPES_i$, indicating buffers do not require a particular (i.e., piperazine) ring to inhibit. Some cyclic group is required, however, because cytoplasmic $taurine_i$ (10 mM) and BES_i (10 mM) were not inhibitory. The stiffness conferred by a ring may be important for inhibition from the inside, but the channel is apparently asymmetric in this respect because extracellular BES had the same effect as $HEPES_o$. The precise length of the alkylsulfonate side chain is not critical because MOPS and MES had similar effects, but the sulfonate group appears to be important because the carboxylic acid 1-piperidinepropionate (10 mM) did not inhibit.

Implications for Future Studies of the Outward Rectifier

It is clear that HEPES-related pH buffers can produce artifacts when studying the properties of the outwardly rectifying anion channel. The most obvious of these is a reduction in single-channel conductance. Inhibition by $HEPES_i$ is particularly strong at physiological (i.e., negative) membrane potentials and is enhanced by raising the temperature from 20 to 37°C. Estimates of channel density will be two- to threefold too high if based on direct comparisons of single-channel conductance in excised patches (bathed with $HEPES_i$) and the macroscopic conductance in intact epithelia. $HEPES_i$ also contributes to outward rectification of the I/V relationship because inhibition is relieved by positive voltage, but it is difficult to predict the effect buffers will have on rate theory models for permeation. When our data were fitted with the simplest model having a single barrier, addition of 10 mM HEPES to both sides increased the apparent height of the barrier at 20°C from 5.22 ± 0.01 kcal/mol to 5.34 ± 0.02 kcal/mol and increased its apparent electrical distance slightly from 34 ± 0.5 to 38 ± 1.4% of the membrane field from the inside, respectively. More accurate barrier models might be affected less by buffers and it should be possible to correct energy calculations for the HEPES effect, but the best approach is probably to use buffers such as TES which do not influence the channel's conductive properties.

HEPES could alter apparent kinetic behavior if the channel can only close when HEPES has dissociated, leading to an overestimate of the mean open time and open probability. It could also affect the

pharmacological properties of the channel by protecting it from reagents and transport inhibitors, some of which are also sulfonates; e.g., 4,4′-diisothiocyanato-2,2′-stilbenedisulfonate (DIDS) and 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonate (SITS). These possibilities were not investigated in this paper. It is unlikely that HEPES would strongly inhibit chloride conductance when cells are maintained in culture medium that contains HEPES because it has somewhat lower potency from the outside and, as mentioned, cell membranes are relatively impermeable to zwitterions. Also, block by external HEPES is partially relieved by membrane potentials typically measured in living cells, and cells may have other mechanisms that compensate for inhibition; for example increasing the number of functional channels.

Other Anion Channels

$HEPES_i$ and $MOPS_i$ induce brief closures and numerous conductance levels in a Cl channel from cultured *Drosophila* neurons (Yamamoto & Suzuki, 1987). While these effects differ qualitatively from those observed in the present study, some differences may be due to temperature; the neuronal experiments were performed at 12°C whereas the present studies were carried out at 20 or 37°C. Low temperature may have slowed $HEPES_i$ interactions sufficiently to allow blocked (and partially blocked) transitions to be resolved. The data of Yamamoto and Suzuki (their Fig. 3, 1987) suggest half-maximal inhibition at approximately 30 mM [$HEPES_i$], which is similar to the value obtained in the present study (23 mM). However, unlike the outward rectifier, HEPES was only effective in blocking the neuronal Cl channel from the cytoplasmic side and its inhibition was strongly voltage dependent at both positive and negative membrane potentials. Apparently not all anion channels are sensitive to substituted taurine buffers; substate conductances of GABA- and glycine-activated channels from spinal neurons are the same when bathed with 5 mM (Hamill, Bormann & Sakmann, 1983) or 10 mM HEPES (Bormann, Hamill & Sakmann, 1987) and they do not rectify, although we note that outward rectification of a GABA-activated channel has been described in hippocampal neurons in HEPES-buffered solutions (Gray & Johnston, 1985). There is evidence that buffers interact with GABA receptors; HEPES competitively inhibits GABA binding to rat brain synaptic membranes with a K_i of 2.25 mM HEPES (Tunnicliff & Smith, 1981).

Buffers that Do Not Inhibit Conductance

None of the N-substituted glycines tested had any significant effect on conductance. Tricine (pK_a = 8.15) would normally be preferred over other glycine buffers (bicine, pK_a = 8.3; glycylglycine, pK_a = 8.4) because its pK_a is nearest the pH of plasma and cytoplasm. Tricine is most effective between pH 7.4 and 8.8, but 10 mM would probably provide enough buffering at pH 7.2 for most situations, and of course its concentration could be increased. One potential disadvantage of tricine is temperature sensitivity ($\Delta pK_a = -0.021$ *vs.* -0.014/°C for HEPES); however, its pK_a is still less temperature-dependent than that of Tris ($\Delta pK_a = -0.031$/°C). Tris itself had no effect on conductance but sometimes caused additional flickering. It was avoided for this reason and because of other undesirable properties such as its ability to permeate membranes and general reactivity. Interestingly, Tris buffer has also been shown to antagonize acetylcholine-activated currents (Wilson, Clark & Pellmar, 1977) by reducing single-channel conductance (Ascher, Marty & Neild, 1978). *Bis*-Tris, which is an effective buffer between pH 5.8–7.2 and is relatively insensitive to temperature ($\Delta pK_a = -0.008$/°C) did not affect conductance or cause noticeable flickering and may be suitable for stabilizing pH in this range.

Which buffers are most suited for studies of the outward rectifier? Of the ones examined here, TES (pK_a = 7.5) is probably the best buffer because it resembles HEPES in most respects but does not inhibit conductance. The glycine-derived buffers and *bis*-Tris also appear compatible when solutions must be buffered outside the pH range 6.8–8.2.

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References

Ascher, P., Marty, A., Neild, T.O. 1978. Lifetime and elementary conductance of the channels mediating the excitatory effects of acetylcholine in *Aplysia* neurones. *J. Physiol. (London)* **278:**177–206

Blatz, A.L., Magleby, K.L. 1984. Ion conductance and selectivity of single calcium-activated potassium channels in cultured rat muscle. *J. Gen. Physiol.* **84:**1–23

Bormann, J., Hamill, O.P., Sakmann, B. 1987. Mechanism of anion permeation through channels gated by glycine and γ-aminobutyric acid in mouse cultured spinal neurones. *J. Physiol. (London)* **385:**243–286

Eisenthal, R., Cornish-Bowden, A. 1974. The direct linear plot. *Biochem. J.* **139:**715–720

Frizzell, R.A. 1987. Cystic fibrosis: A disease of ion channels? *Trends Neurosci.* **10:**190–193

Good, N.E., Izawa, S. 1968. Hydrogen ion buffers. *Methods Enzymol.* **24(Part B):**53–68

Good, N.E., Winget, G.D., Winter, W., Connolly, T.N., Izawa, S., Singh, R.M.M. 1966. Hydrogen ion buffers for biological research. *Biochemistry* **5:**467–477

Gray, R., Johnston, D. 1985. Rectification of single GABA-gated chloride channels in adult hippocampal neurons. *J. Neurophysiol.* **54:**134–142

Halm, D.R., Frizzell, R.A. 1988. Ion permeation through the apical membrane chloride channel in a secretory epithelial cell. *FASEB J.* **2:***A*1283

Halm, D.R., Rechkemmer, G.R., Schoumacher, R.A., Frizzell, R.A. 1988. Apical membrane chloride channels in a colonic cell line activated by secretory agonists. *Am. J. Physiol.* **254:**C505–C511

Hamill, O.P., Bormann, J., Sakmann, B. 1983. Activation of multiple-conductance state chloride channels in spinal neurones by glycine and GABA. *Nature (London)* **305:**805–808

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Hanke, W., Miller, C. 1983. Single chloride channels from *Torpedo* electroplax: Activation by protons. *J. Gen. Physiol.* **82:**25–45

Hanrahan, J.W., Tabcharani, J.A. 1989. Possible role of the outwardly rectifying anion channel in epithelial Cl and HCO_3 transport. *Ann. NY Acad. Sci.* **574:**30–43

Li, M., McCann, J.D., Liedtke, C.M., Nairn, A.C., Greengard, P., Welsh, M.J. 1988. Cyclic AMP-dependent protein kinase opens chloride channels in normal but not cystic fibrosis airway epithelium. *Nature (London)* **331:**358–360

Low, W., Tabcharani, J.A., Hanrahan, J.W. 1990. Outwardly rectifying anion channels: Activation by salts, inhibition by IAA-94 and NPPB. *FASEB J.* **4:**A726

Norris, C.H., Guth, P.S. 1985. Buffers may have pharmacological actions. *Trends Pharmacol. Sci.* **6:**315

Prod'hom, B., Pietrobon, D., Hess, P. 1987. Direct measurement of proton transfer rates to a group controlling the dihydropyridine-sensitive Ca^{2+} channel. *Nature (London)* **329:**243–246

Shoumacher, R.A., Shoemaker, R.L., Halm, D.R., Tallant, E.A., Wallace, R.W., Frizzell, R.A. 1987. Phosphorylation fails to activate chloride channels from cystic fibrosis airway cells. *Nature (London)* **330:**752–754

Sigworth, F.J. 1977. Sodium channels in nerve apparently have two conductance states. *Nature (London)* **270:**265–267

Tabcharani, J.A., Jensen, T.J., Riordan, J.R., Hanrahan, J.W. 1989. Bicarbonate permeability of the outwardly rectifying anion channel. *J. Membrane Biol.* **112:**109–122

Tunnicliff, G., Smith, J.A. 1981. Competitive inhibition of γ-aminobutyric acid receptor binding by N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid and related buffers. *J. Neurochem.* **36:**1122–1126

Vaughan, P.C. 1986. Anion permeability sequence and voltage-clamp measurements in frog muscle at pH 9. *Can. J. Physiol. Pharmacol.* **64:**A28

Vergara, C., Latorre, R. 1983. Kinetics of Ca^{2+}-activated K^+ channels from rabbit muscle incorporated into planar bilayers. *J. Gen. Physiol.* **82:**543–568

Wilson, W.A., Clark, M.T., Pellmar, T.C. 1977. Tris buffer attenuates acetylcholine responses in *Aplysia* neurons. *Science* **196:**440–441

Witte, O.W., Speckmann, E.-J., Walden, J. 1985. Acetylcholine responses of identified neurons in *Helix pomatia*. II. Pharmacological properties of acetylcholine responses. *Comp. Biochem. Physiol.* **80:**C25–35

Woodhull, A.M. 1973. Ionic blockage of sodium channels in nerve. *J. Gen. Physiol.* **61:**687–708

Yamamoto, D., Suzuki, N. 1987. Blockage of chloride channels by HEPES buffer. *Proc. R. Soc. London B* **230:**93–100

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