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MEMBRANE RESPONSES EVOKED BY ORGANIC BUFFERS IN IDENTIFIED LEECH NEURONES

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Summary

Using single-electrode current-clamp and two-electrode voltage-clamp, different identified neurones of the leech (*Hirudo medicinalis* L.) were shown to hyperpolarize, or to exhibit outward currents (usually >2 nA), during bath application of the organic buffers Mops, Pipes and Mes (10 mmol l^{-1}). Tris and Hepes had little or no effect on the membrane properties of neurones when they were added to a bathing saline buffered by CO_2–HCO_3^- at a constant pH of 7.4.

Outward currents evoked by buffers obtained from two suppliers, Roth and Sigma, were not significantly different, except in the case of Pipes. Steady-state currents evoked by Pipes supplied by Roth were eightfold larger than steady-state currents evoked by Pipes from Sigma. A transient current peak always present in responses evoked by Pipes from Roth was never present in responses evoked by Pipes from Sigma.

Outward currents evoked by buffers were due to a conductance increase and appeared to be carried by Cl^-. In low-Cl^- saline, hyperpolarizations evoked by Mops in Retzius cells were reduced. When Cl^- was injected into heart motor neurones, the hyperpolarizations evoked by Mops reversed. Tetraethylammonium (TEA^+) injected into heart interneurones did not block Mops-evoked hyperpolarizations.

Mops-evoked outward currents in Retzius cells were partially blocked by *d*-tubocurarine and bicuculline methiodide, and the latter also partially blocked Mops-evoked hyperpolarizations in HE cells. Since *d*-tubocurarine partially blocked acetylcholine-evoked Cl^- currents in Retzius cells and bicuculline methiodide partially blocked carbachol-evoked hyperpolarizations in HE cells, Mops appears to act on the cholinergic receptors of these neurones by mediating a Cl^- conductance.

Key words: Cl^- conductance, Mops, Hepes, Tris, Mes, buffer, bicuculline, *d*-tubocurarine, leech neurones, *Hirudo medicinalis*.

Introduction

Organic pH buffers have been used routinely in physiology for several decades (Good *et al.* 1966). In particular, the zwitterionic aminosulphonic acids Hepes, Mops and Pipes are widely used to stabilize pH between 6.1 and 8.2. Hepes is probably the commonest organic buffer to be used in physiological salines; with a pK value close to 7.5, it is ideally suited to buffer salines at pH values between 7.2 and 7.5, which are the values found in the blood of most terrestrial and freshwater animals (Burton, 1975). When Good *et al.* (1966) introduced Hepes, Pipes, Mes and other organic buffers, they were aware of possible side-effects and such side-effects of organic buffers have been described. For example, Hepes has been shown to block Cl^- channels in neurones of *Drosophila melanogaster* (Yamamoto and Suzuki, 1987) and Hepes and Mes both affect K^+ currents in squid giant axon (Wanke *et al.* 1979). It appears, however, that these organic buffers are often assumed to be largely inert in the presence of biological structures such as cell membranes and to have no effect on physiological processes.

While studying the effects of external pH on the membrane properties of neurones in segmental ganglia of the leech, we observed that rather small external pH changes (7.4 and 7.2–7.0, adjusted by Mops) had a substantial effect on the membrane potential and firing rate of heart excitor motor neurones and heart interneurones (Mangold, 1995). We then tested different buffers at constant pH to see whether the buffers themselves could be having an effect and found that all leech neurones tested responded to the organic buffers most commonly used in physiological experiments. Changes in external pH had little or no effect. This contrasts with the results of previous studies, where significant changes in ion conductances induced by external pH changes have been reported (Konnerth *et al.* 1987; Jarolimek *et al.* 1989; Gottmann *et al.* 1989). The present study describes the action of a variety of commonly used buffers on identified leech

neurones and the underlying mechanism of these buffer effects. The results show that Mops, Pipes and Mes, but not Hepes and Tris, induced outward currents apparently due to an increased membrane conductance to Cl^-.

Materials and methods

Leeches (*Hirudo medicinalis* L.) were obtained from Moser Blutegelhandel (Schorndorf, Germany) and kept in fresh water at 16–18 °C. The animals were kept for 3–7 days at room temperature (20–25 °C) before experimentation. Animals were anaesthetized in ice-cold water before dissection. Individual segmental ganglia were removed from the animals and pinned ventral side up in a Petri dish or Perspex chamber lined with Sylgard (Dow-Corning). The ventral ganglionic capsule overlying the nerve cell bodies was removed immediately before the experiments.

Ganglia were continuously superfused with saline based either on a CO_2–HCO_3^- buffer or on a Tris buffer. The composition of the CO_2–HCO_3^--based saline was (in $mmol\,l^{-1}$): NaCl, 61; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; $NaHCO_3$, 25; sucrose, 10. The saline was continuously bubbled with 5 % CO_2/95 % O_2. The organic buffers were tested at a concentration of $10\,mmol\,l^{-1}$ (replacing $10\,mmol\,l^{-1}$ sucrose): Tris (lot no. 24H5710) was obtained from Sigma, Germany, and Hepes (lot no. 42420360), Mops (lot no. 39211637) and Pipes (lot no. 29315466) were all obtained from Roth, Germany. The pH of all solutions was adjusted to 7.4 with HCl or NaOH using a 654 pH meter from Metrohm, Switzerland.

The composition of the Tris-based saline (in $mmol\,l^{-1}$) was: NaCl, 75; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; Tris, 10; sucrose, 23. pH was adjusted to 7.4 with $8\,mmol\,l^{-1}$ HCl. The buffers to be tested, Hepes, Mops, Pipes and Mes (lot no. 3386479), were added at a concentration of $10\,mmol\,l^{-1}$ after removing equimolar amounts of sucrose. Since all these buffers are acids, the pH of the solutions was adjusted to 7.4 by adding NaOH. In one set of experiments, we used Hepes (lot no. 64H5728), Mops (lot no. 41H5628), Mes (lot no. 103H5702) and Pipes (lot no. 14H5733) from Sigma Chemical Company, Germany. When Na^+-free solutions were used, we substituted *N*-methyl-*d*-glucamine for Na^+.

In one set of experiments, we used different concentrations of Mops (2, 10 and $20\,mmol\,l^{-1}$) in a Tris-based saline containing $65\,mmol\,l^{-1}$ NaCl and with pH adjusted to 7.4 by the addition of NaOH. Sucrose was added to the solutions containing 2 and $10\,mmol\,l^{-1}$ Mops so that all three solutions had the same osmotic strength. *d*-Tubocurarine and bicuculline methiodide (Sigma, Germany) were used in some experiments. In some experiments, a low-Cl^- saline was used. This was a Tris-based saline in which all Cl^--containing compounds other than $CaCl_2$ were replaced by SO_4^{2-}-containing compounds. pH was adjusted using H_2SO_4.

The different organic buffers were added directly into the saline to give final concentrations of $10\,mmol\,l^{-1}$ and were applied to the preparations for 1.5–4 min. The effects of buffers were tested on a variety of identified cells: Retzius cells; motor neurones, consisting of heart excitor (HE), annulus erector (AE) and the excitor of the ventrolateral circular muscles (CV); three types of mechanosensory cell, P (pressure), T (touch) and N (noxious); heart interneurones (HN); and anterior pagoda (AP) cells (Muller *et al.* 1981). Neuronal cell bodies were penetrated with conventional microelectrodes filled with $3\,mol\,l^{-1}$ potassium acetate. The pH of the potassium acetate solution was adjusted to 7.4 by adding approximately $20\,mmol\,l^{-1}$ HCl. Electrode resistances measured in physiological saline were 30–40 MΩ. In some experiments, electrodes were filled with $1.8\,mol\,l^{-1}$ tetraethylammonium acetate (TEA^+) and $2\,mol\,l^{-1}$ potassium acetate; pH was adjusted to 7.4 by adding HCl. In one set of experiments, neurones were loaded with Cl^- using microelectrodes filled with $3\,mol\,l^{-1}$ KCl. Cl^- either leaked into the cells or was introduced by injection of negative current.

An Axoclamp 2A amplifier (Axon Instruments, USA) was used in bridge mode for conventional intracellular recording, in DCC mode (discontinuous current-clamp) for current-clamp experiments or in TEVC mode (two-electrode voltage-clamp) for voltage-clamp experiments. Sample rate in DCC mode was typically 2 kHz. All measurements were made relative to the bath potential to avoid voltage offsets when the bath solution was changed.

Data were recorded on a DAT recorder (Biologic, France) for later playback on a chart recorder (Yokogawa, ORP 1200). Some voltage-clamp data (see Fig. 2) were recorded on-line on chart paper (Linseis, L6534) and sampled on a personal computer using an 18.2 Hz per channel A/D board, controlled by SENTRY software (U. Lönnendonker, Universität Kaiserslautern, Germany).

All measurements are given as mean values ± standard deviation (S.D.); *N* indicates the number of experiments. Data were statistically analyzed using a modified two-tailed *t*-test after Dixon and Massey (1969).

Results

Tris and Hepes are probably the most commonly used organic buffers in physiological experiments, including those carried out in the central nervous system of the leech. We therefore tested the effects of Hepes and Tris in a CO_2–HCO_3^--buffered saline. In Retzius cells, voltage-clamped to −50 mV, bath application of Tris and Hepes (both $10\,mmol\,l^{-1}$) had little or no effect (Fig. 1). Currents evoked by Tris were −0.2±0.6 nA (*N*=3) and currents evoked by Hepes were 0.21±0.25 nA (*N*=8). In contrast, Mops evoked outward currents of 1.36±0.5 nA (*N*=8).

These results were confirmed in current-clamp experiments. When Hepes ($10\,mmol\,l^{-1}$) was added to a Tris-buffered saline, no significant effect on membrane potential, action potential frequency or input resistance of Retzius neurones was seen (Fig. 2). Mops, Mes and Pipes ($10\,mmol\,l^{-1}$) affected all three parameters: these buffers hyperpolarized the membrane (Fig. 2A), leading to a suppression of action potentials, and they decreased the input resistance (Fig. 2B). The mean

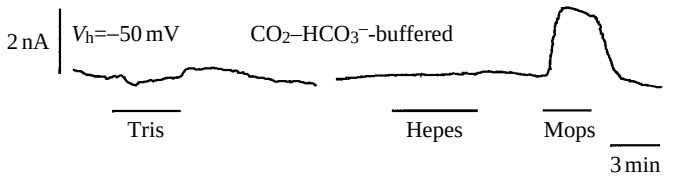

Fig. 1. Two-electrode voltage-clamp of Retzius neurones. Holding potential (V_h) was −50 mV. Standard buffer was CO_2–HCO_3^-. Bath application of 10 mmol l^{-1} Tris and Hepes had no significant effects on the holding current. Bath application of 10 mmol l^{-1} Mops evoked an outward current.

membrane hyperpolarizations caused by these buffers in Retzius neurones were in the region of −15 mV (Table 1). While the membrane hyperpolarizations evoked by Mops and Mes were readily reversible, within 1–2 min of the removal of these buffers, the effects of Pipes were longer-lasting (Fig. 2). Even 10 min after the removal of Pipes, the membrane potential had not fully returned to its initial value. Changing the pH of Mops-buffered saline to 7 had no influence on the Mops-induced membrane hyperpolarization.

Hyperpolarizing responses to these buffers were also recorded in N, P (Table 1) and T sensory neurones, in AE, HE (Table 1) and CV motor neurones and in the HN interneurone (data not shown). None of these neurones responded to Hepes.

Mops, added at different concentrations between 2 and 20 mmol l^{-1}, hyperpolarized the Retzius neurones in a concentration-dependent manner (Fig. 3). While 2 mmol l^{-1} Mops evoked a slow and small hyperpolarization of 3.4±1.8 mV (N=4) within 2 min, 10 mmol l^{-1} Mops increased this to a hyperpolarization of −14±3 mV (N=4) and 20 mmol l^{-1} Mops gave a hyperpolarization of 16±2 mV (N=3). The new steady states were obtained within 1 min at these higher Mops concentrations, with a slightly faster time course in the presence of 20 mmol l^{-1} Mops.

Table 1. *Membrane potentials (E_m) recorded in four different cell types in the presence of different buffers*

	Membrane potential (mV)			
Buffer	Retzius cell	P cell	HE cell	T cell
Tris	–50.8±3.7	–47.8±4.2	–38.7±3.4	–41.7±9
Mops	–65.8±3.1	–51.2±6.2	–54.1±7.7	–51±12
Tris–Mops	–15±2.6	–3.4±2.3	–14.7±4.2	–9.3±3.1
	(6)	(5)	(11)	(3)
Tris	–49±2.2	–45±3.9	–41, –37	–41.7±8.1
Mes	–64±1	–46.7±4	–51, –48	–50.3±9.1
Tris–Mes	–15.8±2.6	–1.7±0.8	–10, –11	–8.6±2.1
	(4)	(5)	(2)	(3)
Tris	–48.3±1.2	–42.7±3.2	–37	
Pipes	–65±1	–45.5±3.5	–60	
Tris–Pipes	–16.7±0.6	–2.5±1.8	–23	
	(3)	(3)	(1)	

Values in Tris-buffered saline and during bath application of Mops, Mes or Pipes are given as means ± S.D. (N) in mV.

The difference potentials between E_m in Tris and E_m in the test solutions were calculated.

In all experiments, buffers were applied in the order Mops, Mes, Pipes.

Membrane potentials measured in Tris-buffered saline were not combined as a single value but are given separately, as the level before the application of the respective buffer, because the E_m of cells usually decayed during the course of an experiment.

Comparison of buffers from different suppliers: effects on membrane conductance

To obtain a quantitative measure of the changes in membrane conductance, we voltage-clamped Retzius cells at −50 mV and stepped the holding potential to −51.5 mV or −55 mV while applying buffers (10 mmol l^{-1}) from different

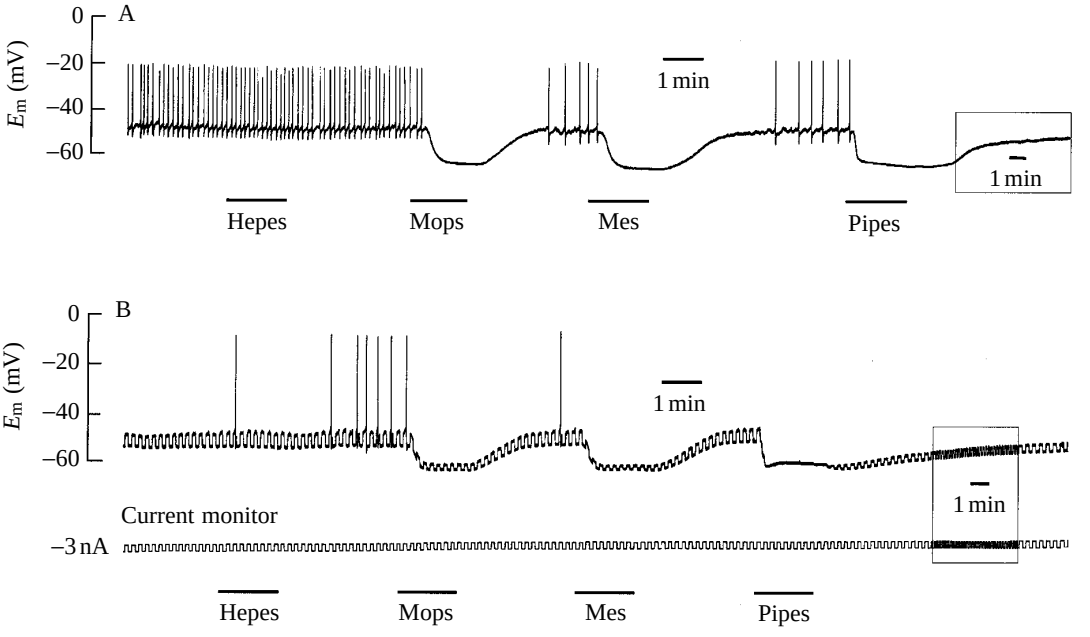

Fig. 2. Examples of intracellular recordings from Retzius neurones. Standard buffer was Tris. (A) Bath application of 10 mmol l^{-1} Mops, Mes or Pipes hyperpolarized the cell. 10 mmol l^{-1} Hepes had no effect. (B) Pulses of negative current (−3 nA) were injected into a Retzius neurone. The amplitudes of these hyperpolarizations decreased during application of 10 mmol l^{-1} Mops, Mes or Pipes, indicating a conductance increase. E_m, membrane potential.

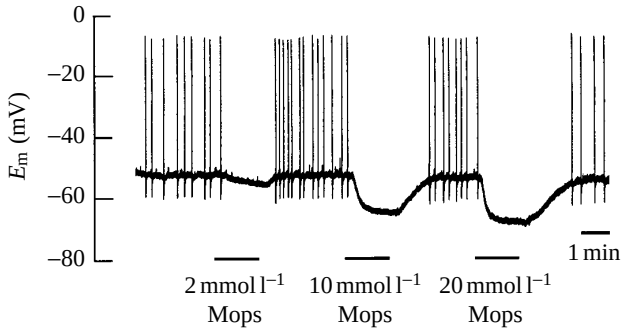

Fig. 3. An intracellular recording from a Retzius neurone. Standard buffer was Tris. Bath application of increasing concentrations of Mops caused an increase in response amplitude and an increase in the rate of decrease of membrane potential.

suppliers. Fig. 4A shows outward currents evoked by buffers obtained from Roth. Mops evoked currents of 2.7±1.02 nA (*N*=9) and Mes evoked currents of 4.1±0.67 nA (*N*=9). Pipes always caused a distinct peak in the outward current (15.5±6.54 nA, *N*=5) before it reached a plateau of 4.8±1.74 nA (*N*=8). The membrane conductance of Retzius cells was 72.9±21.7 nS (*N*=8) before the application of buffers. The conductance increased 3.2±1.7-fold in the presence of Mops (*N*=8), 5.0±2.0-fold in the presence of Mes (*N*=8) and 30±13.1-fold in the presence of Pipes (*N*=6), measured during the plateau phase of the response.

Hepes obtained from Sigma had no effect on membrane properties. The application of Mops and Mes from Sigma evoked currents whose amplitudes and conductances were not significantly different from those evoked by buffers from Roth ($P>0.1$; Fig. 4B). In contrast, Pipes from Sigma evoked much smaller currents (0.6±0.1 nA, *N*=3) than Pipes from Roth. A peak in the outward current was never observed and the conductance increase was only 4.6±1.6-fold (*N*=3).

Ion-dependence of the Mops-induced conductance increase

These experiments were carried out with Mops buffer on Retzius and on HE cells. Initially, the possibility of a Cl^- conductance increase in Retzius cells induced by Mops was studied. Recordings were made from Retzius cells under current-clamp conditions while the cells were bathed in low-Cl^- saline (4 mmol l^{-1} instead of the 93 mmol l^{-1} present in normal saline). Low-Cl^- saline induced bursts of action potentials, but had no effect on the baseline membrane potential. 1 min after application of low-Cl^- saline, Mops was applied for 1.5 min (Fig. 5). The hyperpolarization evoked by Mops under these conditions had an amplitude of 2.5±2.29 mV (*N*=3), which was much smaller than the amplitude of 15±2.6 mV (*N*=6) evoked in normal saline. This result indicates that the current evoked by Mops in Retzius cells is likely to be carried mainly by Cl^-.

We confirmed this result using HE cells, which were impaled with microelectrodes filled with 3 mol l^{-1} KCl. Chloride ions were allowed to diffuse into the cells until they

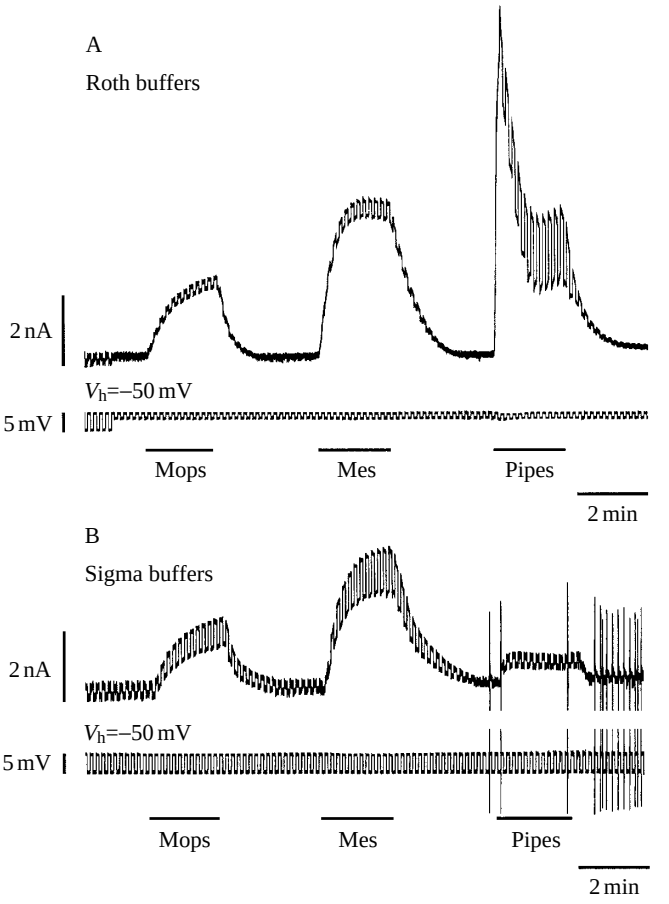

Fig. 4. Two-electrode voltage-clamp of Retzius neurones. Standard buffer was Tris. The holding current (V_h) was stepped periodically from −50 mV to −51.5 mV or −55 mV and caused corresponding membrane currents. (A) Bath application of 10 mmol l^{-1} Mops, Mes or Pipes (obtained from Roth) evoked outward currents and increased the membrane conductance. (B) Bath application of 10 mmol l^{-1} Mops, Mes or Pipes (obtained from Sigma) evoked outward currents and increased the membrane conductance. The changes following application of Pipes were much smaller than those obtained when Pipes from Roth was used.

were so loaded that the direction of potential changes based on Cl^- conductances was reversed (Kerkut and Thomas, 1964). Approximately 1 min after insertion of a KCl-filled microelectrode into an HE cell, the hyperpolarizing inhibitory postsynaptic potentials (IPSPs) reversed to depolarizing synaptic potentials (Fig. 6A, inset). Since the IPSPs are due to a transmitter-induced Cl^- conductance (Thompson and Stent, 1976), their reversal indicates a shift of the Cl^- equilibrium potential to values more positive than the actual membrane potential. Under these conditions, the membrane was reversibly depolarized by Mops (Fig. 6A), on average by 5.1±5.3 mV (*N*=6), and the cells stopped firing action potentials, presumably because the voltage-dependent Na^+ channels were inactivated.

The KCl-filled microelectrode was then withdrawn from the cell and replaced by a microelectrode filled with potassium

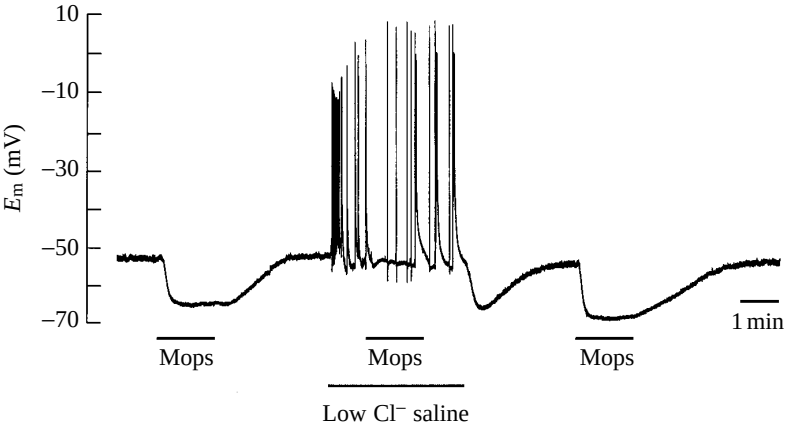

Fig. 5. Intracellular recording from a Retzius neurone. Standard buffer was Tris. In low-Cl^- saline, the response upon bath application of Mops was greatly reduced.

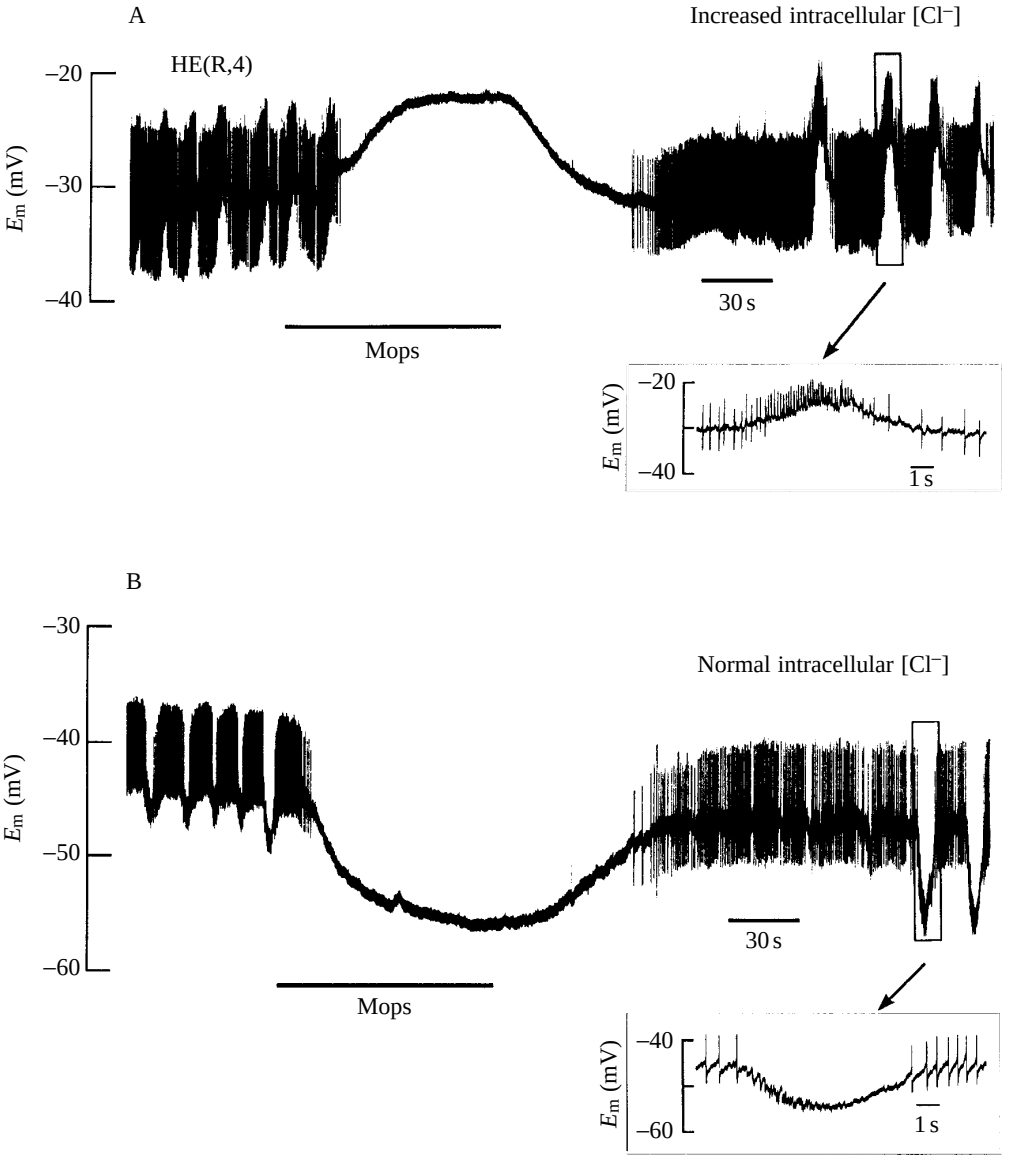

Fig. 6. Intracellular recording from a heart interneurone in the fourth segmental ganglion, HE(R,4). Standard buffer was Tris. (A) Recording with a $3\,mol\,l^{-1}$ KCl electrode. A normally hyperpolarizing response during bath application of $10\,mmol\,l^{-1}$ Mops was reversed after Cl^- had been injected into the cell. Bursts of action potentials were interrupted by reversed IPSPs (inset) caused by a postsynaptic heart interneurone. (B) A recording from the same neurone with a $4\,mol\,l^{-1}$ potassium acetate electrode 5 min after removal of the KCl electrode. The neurone now exhibited its normal rhythmic activity. The inset shows normal hyperpolarizing IPSPs. Bath application of $10\,mmol\,l^{-1}$ Mops caused a hyperpolarizing response.

acetate, as used in all other experiments. The same cell now showed a normal bursting pattern, and the IPSPs were again hyperpolarizing (Fig. 6B, inset). Application of Mops now evoked the usual membrane hyperpolarization of around −15 mV, as described above. These experiments indicate that Mops induced a Cl^--dependent membrane potential change.

This suggestion is in line with the observation that injection into heart interneurones of TEA^+, which blocks K^+ currents in these cells (Simon *et al.* 1992), did not reduce Mops-induced hyperpolarizations (data not shown). Heart interneurones were impaled with tetraethylammonium acetate electrodes and the broadening of action potentials and the reduction of the undershoot indicated the presence of intracellular TEA^+ in our experiments.

We also observed the effect of loading Retzius cells with Cl^- by injecting negative current. This treatment reduced the hyperpolarizations but did not reverse them. This observation might be due to the large size of the Retzius cells and their ability to regulate their low intracellular Cl^- concentration effectively (Munsch and Schlue, 1993), which would prevent sufficient Cl^- loading.

Antagonists of the Mops responses

We tested the effects of antagonists known to affect transmitter-induced Cl^- conductances on the Mops-induced Cl^--dependent membrane response. *d*-Tubocurarine, which partly inhibits the hyperpolarizing response elicited by acetylcholine in Retzius neurones (Szczupak *et al.* 1993), reduced the Mops-induced outward current in voltage-clamped Retzius cells (Fig. 7A). The mean reduction was 43±8 % (*N*=4) in the presence of 0.5 mmol l^{-1} *d*-tubocurarine. An effect was only seen when the drug was applied several minutes (4–6 min) before addition of the buffer. After removal of *d*-tubocurarine, there was a full recovery of the Mops-induced outward current (Fig. 7A).

Bath application of bicuculline methiodide (0.1 mmol l^{-1}) partially blocks IPSPs and hyperpolarizations evoked by application of the acetylcholine agonist carbachol in HE cells (Schmidt and Calabrese, 1992). The same concentration of bicuculline methiodide reduced Mops-evoked membrane hyperpolarizations by 74±9 % (*N*=5; data not shown). These results on Retzius cells and HE cells indicate that Mops might act on cholinergic receptors.

Mops-induced outward currents in Retzius cells were inhibited by 43±12 % (*N*=4) in the presence of 0.1 mmol l^{-1} bicuculline methiodide and by 83±9 % (*N*=3) in the presence of 0.25 mmol l^{-1} bicuculline methiodide (Fig. 7B). In Retzius cells, the addition of bicuculline itself induced small outward currents of 0.31±0.15 nA (*N*=4) at a concentration of 0.1 mmol l^{-1} and of 0.6±0.14 nA (*N*=3) at a concentration of 0.25 mmol l^{-1}. This effect and the reduction of the membrane responses evoked by Mops were both fully reversible after the removal of bicuculline.

Discussion

Characterization of the responses to application of the buffers

The buffers Hepes and Tris had little or no effect on the membrane properties of leech neurones, but application of Mops, Pipes and Mes led to hyperpolarizing responses in all neurones from which recordings were made. In Retzius cells, these hyperpolarizations were associated with a conductance increase, indicating an efflux of K^+ and/or an influx of Cl^-. Retzius cells, when bathed in low-Cl^- saline, exhibited an 83 % reduction in the hyperpolarizations evoked by Mops. When HE cells were loaded with Cl^-, the hyperpolarizing response obtained in response to the application of Mops was reversed. Injection of TEA^+ into heart interneurones, which effectively blocks K^+ currents in these cells (Simon *et al.* 1992), did not reduce Mops-induced hyperpolarizations. These results suggest that Cl^- influx is the main mechanism leading

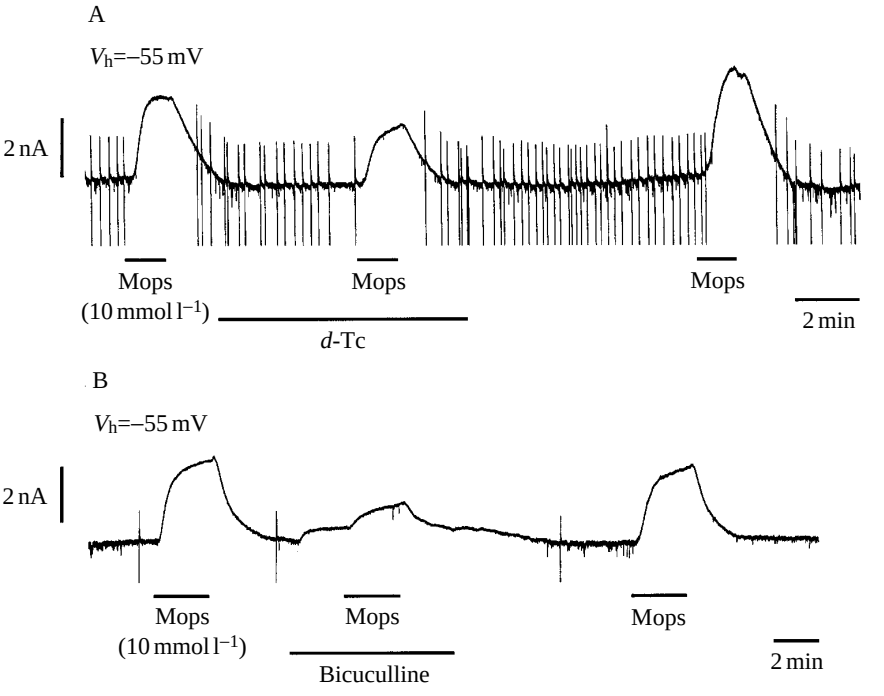

Fig. 7. Two-electrode voltage-clamp of Retzius neurones. Holding potential (V_h) was −55 mV. Standard buffer was Tris. An outward current evoked by bath application of 10 mmol l^{-1} Mops was partially blocked in the presence of (A) 0.5 mmol l^{-1} *d*-tubocurarine (*d*-Tc) and (B) 0.25 mmol l^{-1} bicuculline methiodide.

to the membrane hyperpolarizations or outward currents evoked by Mops application.

This suggestion is supported by the observation that the membrane potential (E_m) measured in Retzius cells and P cells when Mops was present was always near the equilibrium potential for Cl^- (E_{Cl}). The E_m values of Retzius cells during the application of Mops, and of Mes and Pipes, were not significantly different ($P>0.05$) from the E_{Cl} of -61.7 ± 9.1 mV, measured with ion-sensitive microelectrodes in these cells (Munsch *et al.* 1995). In P cells, Mops hyperpolarized the membrane to values that were not significantly different from the measured E_{Cl} of -56.6 ± 6.1 mV (Munsch *et al.* 1995).

In HE cells, we measured an E_m of -54.1 ± 7.7 mV ($N=11$) in the presence of Mops. This value is close to the mean reversal potential of -53 mV for Cl^--mediated hyperpolarizations elicited by focal application of the acetylcholine (ACh) agonist carbachol onto the somata of HE cells (Schmidt and Calabrese, 1992). However, Cl^--mediated IPSPs in HE cells (Calabrese, 1979) were reported to reverse at membrane potentials of approximately -70 mV (Nicholls and Wallace, 1978). Since all measurements were made in the cell bodies, this discrepancy might be due to an uneven distribution of Cl^-, with a higher concentration in the cell body than in the neurites, as has been previously suggested (Schmidt and Calabrese, 1992).

The Mops-induced currents appear to be mainly carried by Cl^-, and this is also likely to be true of those induced by Mes and Pipes. However, a comparison of the current amplitudes evoked by Mops, Mes and Pipes revealed that other mechanisms may also be involved. Current amplitudes evoked by Mops (2.7 ± 1.02 nA) were significantly smaller ($P<0.01$) than those evoked by Mes. Conductance changes, however, were not significantly different for the two buffers ($P>0.05$). One mechanism leading to the smaller current amplitude in Mops could be the down-regulation of an inward current together with the development of an outward current. The amplitudes of the plateau phases of the currents evoked by Pipes (Roth) were not significantly different from the current amplitudes evoked by Mes. In contrast, the conductances after application of Pipes were about six times as large, indicating that Pipes might also evoke an inward current that antagonizes the outward current. A delayed onset of this antagonizing inward current would be indicated by the peak observed in the outward current at the beginning of the response upon application of Pipes.

The outward current in Retzius cells evoked by bath application of Mops was partially blocked by *d*-tubocurarine, a known blocker of nicotinic receptors. Szczupak *et al.* (1993) showed that *d*-tubocurarine partially blocked Cl^--mediated outward currents evoked by focal application of ACh onto the somata of Retzius cells. The block of both responses by *d*-tubocurarine indicates that Mops might lead to a Cl^- conductance in Retzius cells by acting on cholinergic receptors. In HE cells, Mops also appears to act on cholinergic receptors, since hyperpolarizations evoked by bath application of Mops were partially blocked by bicuculline methiodide. This antagonist has also been shown to block Cl^--mediated hyperpolarizations in HE cells evoked by focal application of carbachol onto the cell somata (Schmidt and Calabrese, 1992).

The outward currents in Retzius cells evoked by Mops were also partially blocked in the presence of bicuculline methiodide (Fig. 7B). It is not known whether the ACh-evoked outward currents in Retzius cells are bicuculline-sensitive. However, blocking of ACh responses by bicuculline has been reported before. For example, bicuculline chloride blocks Cl^- responses to the application of ACh in R2 neurones of *Aplysia* (Yarowsky and Carpenter, 1978) and it has been shown to block depolarizing responses to ACh in unidentified neurones of the locust (Benson, 1988). In addition, bicuculline blocks cholinergic IPSPs and depolarizations evoked by focal application of ACh in GI 2 neurones of the cockroach *Periplaneta americana* (Buckingham *et al.* 1994).

There is, however, still some uncertainty about the identity of the receptor. Carpenter *et al.* (1977) showed that, in identified neurones of *Aplysia*, *d*-tubocurarine blocks the membrane responses from Na^+- and Cl^--dependent conductances, irrespective of whether the responses were evoked by ACh, GABA, dopamine, octopamine or other putative transmitters. The authors concluded that *d*-tubocurarine is not a specific nicotinic blocker, but rather blocked a whole class of receptor-activated Na^+- and Cl^--dependent responses. In addition, focal application of ACh onto the somata of P and N cells only evoked depolarizing responses (Sargent *et al.* 1977), but both cell types responded with a hyperpolarization during bath application of Mops, Mes or Pipes. However, hyperpolarizing cholinergic responses to buffer application might be evoked at dendritic processes equipped with a different set of receptors and/or ionic gradients (see above).

Side-effects of buffers

The side-effects of buffers depend on the preparation; they are not restricted to the leech, nor are they limited to Mops, Mes or Pipes. Hepes, for example, which had no effects in the leech, blocks Cl^- channels in neurones of *Drosophila melanogaster* when the cytoplasmic side of the membrane is exposed to the buffer (Yamamoto and Suzuki, 1987). In the giant axon of the squid *Loligo vulgaris*, Hepes and Mes alter K^+ currents (Wanke *et al.* 1979), and on afferent units in the ampullar nerve of the semicircular canal in frogs, Hepes appears to act as an antagonist of ACh (Norris and Guth, 1985). Tris, which has no side-effects in the leech, attenuates ACh responses in some *Aplysia californica* neurones (Wilson *et al.* 1977). The modulatory effects of intracellular pH on K^+ currents in human lymphocytes were unaffected by Hepes, Mes and Taps (Deutsch and Lee, 1989). However, a significantly lower average current was observed in Tris than in a control at the same pH. In medial giant axons of the crayfish *Procambarus clarkii*, Mes seemed to enhance a Cl^- permeability at acidic pH (Strickholm, 1981).

In other preparations, organic buffers appear to have no side-

effects. For example, the decrease in the size of miniature endplate currents at acidic pH in frog neuromuscular junctions did not depend on the presence of Mes and Mops, the buffers used by Landau *et al.* (1981). Pasternack *et al.* (1992) replaced Tris and Pipes by Hepes when studying the influence of pH on GABA-gated Cl^- conductances in crayfish muscle fibres and no influence of these buffers was observed. In CA1 neurones of rat hippocampus, no detectable currents were evoked by Hepes, Mops, Pipes or Mes (K. H. Backus, unpublished observations).

Great care should be taken when using a Mops-, Pipes- and Mes-buffered saline for electrophysiological experiments on leech neurones because the outward currents evoked by these buffers might lead to misinterpretation of the experimental results. The hyperpolarizations of Retzius cell membranes measured upon lowering pH to 7.0 (Deitmer, 1991) were presumably due to the presence of Mops rather than to the acidic pH. Gottmann *et al.* (1989) described a proton-induced Cl^- current in Retzius neurones; our results suggest that this outward current was evoked by Pipes, rather than by the low external pH, since lowering pH to 7.0 or even 6.4 in Tris-buffered saline does not appear to affect the membrane properties of Retzius cells (J. Schmidt, unpublished observation). Frey and Schlue (1993) observed a small depolarization of Retzius neurones in the presence of saline containing 10 mmol l^{-1} Pipes at pH 6.4. This can probably be explained by the fact that KCl electrodes were used in their experiments and enough Cl^- might have leaked into the cell to reverse the driving force for Cl^-.

The effects of buffers on membrane properties demonstrated here point to the importance of checking drugs for possible side-effects. It is noteworthy that a substance sold under the same name (Pipes) by different companies and synthesized by different producers (company information) has qualitatively and quantitatively different effects on membrane properties. The differing effects of Pipes from different suppliers may indicate that the observed effects were caused not by the buffers themselves but by impurities introduced during their production.

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References

BENSON, J. A. (1988). Bicuculline blocks the response to acetylcholine and nicotine but not to muscarine or GABA in isolated insect neuronal somata. *Brain Res.* **458**, 65–71.

BUCKINGHAM, S. D., HUE, B. AND SATTELLE, D. B. (1994). Actions of bicuculline on cell body and neuropilar membranes of identified insect neurones. *J. exp. Biol.* **186**, 235–244.

BURTON, R. F. (1975). *Ringer Solutions and Physiological Salines*. Bristol: Wright Scientechnica.

CALABRESE, R. C. (1979). The roles of endogenous membrane properties and synaptic interaction in generating the heartbeat rhythm of the leech, *Hirudo medicinalis*. *J. exp. Biol.* **82**, 163–176.

CARPENTER, D. O., SWANN, J. W. AND YAROWSKY, P. J. (1977). Effect of curare on responses to different putative neurotransmitters in *Aplysia* neurones. *J. Neurobiol.* **8**, 119–132.

DEITMER, J. W. (1991). Electrogenic sodium-dependent bicarbonate secretion by glial-cells of the leech central nervous system. *J. gen. Physiol.* **98**, 637–655.

DEUTSCH, C. AND LEE, S. C. (1989). Modulation of K^+ current in human lymphocytes by pH. *J. Physiol., Lond.* **413**, 399–413.

DIXON, W. J. AND MASSEY, F. J. (1969). *Introduction to Statistical Analysis*, 3rd edn. New York: McGraw Hill.

FREY, G. AND SCHLUE, W.-R. (1993). pH recovery from intracellular alkalinization in Retzius neurones of the leech central nervous system. *J. Physiol., Lond.* **462**, 627–643.

GOOD, N. E., WINGET, G. D., WINTER, W., CONOLLY, T. N., IZAWA, S. AND SINGH, R. M. M. (1966). Hydrogen ion buffers for biological research. *Biochemistry, N.Y.* **5**, 467–477.

GOTTMANN, K., DIETZEL, I. D. AND LUX, H. D. (1989). Proton-induced chloride current and voltage activated Na^+ and Ca^{2+} current in embryonic neurones from the medicinal leech (*Hirudo medicinalis*). *Neurosci. Lett.* **96**, 173–178.

JAROLIMEK, W., MISGELD, U. AND LUX, H. D. (1989). Activity dependent alkaline and acid transients in guinea pig hippocampal slices. *Brain Res.* **505**, 225–232.

KERKUT, G. A. AND THOMAS, R. C. (1964). The effect of anion injection and changes in the external potassium and chloride concentration on the reversal of IPSP and acetycholine. *Comp. Biochem. Physiol.* **11**, 199–213.

KONNERTH, A., LUX, H. D. AND MORAD, M. (1987). Proton-induced transformation of calcium channel in chick dorsal root ganglion cells. *J. Physiol., Lond.* **386**, 603–633.

LANDAU, E. M., GAVISH, B., NACHSHEN, D. A. AND LOTAN, I. (1981). pH dependence of the acetylcholine receptor channel. *J. gen. Physiol.* **77**, 647–666.

MANGOLD, C. (1995). Einfluß extrazellulärer pH – Veränderungen und organischer Puffer auf die Aktivität des Herzschlag kontrollierenden neuronalen Netzwerks des Blutegels *Hirudo medicinalis*. Diploma thesis, University of Kaiserslautern, Germany.

MULLER, K. J., NICHOLLS, J. G. AND STENT, G. S. (1981). *Neurobiology of the Leech*, pp. 256–257. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

MUNSCH, T., REUSCH, M. AND DEITMER, J. W. (1995). Intracellular chloride activity of leech neurones and glial cells in physiological, low chloride saline. *J. comp. Physiol.* A **176**, 273–280.

MUNSCH, T. AND SCHLUE, W.-R. (1993). Intracellular chloride activity and the effect of 5-hydroxytryptamine on the chloride conductance of leech Retzius neurones. *Eur. J. Neurosci.* **5**, 1551–1557.

NICHOLLS, J. G. AND WALLACE, B. G. (1978). Modulation of transmission at an inhibitory synapse in the central nervous system of the leech. *J. Physiol., Lond.* **281**, 157–170.

NORRIS, C. H. AND GUTH, P. S. (1985). Buffers may have pharmacological actions. *Trends physiol. Sci.* **6**, 315.

PASTERNACK, M., BOUNTRA, C., VOIPIO, J. AND KAILA, K. (1992). Influence of extracellular and intracellular pH on GABA-gated chloride conductance in crayfish muscle fibers. *Neuroscience* **47**, 921–929.

SARGENT, P. B., KING-WAI, Y. AND NICHOLLS, J. G. (1977). Extrasynaptic receptors on cell bodies of neurones in central nervous system of the leech. *J. Neurophysiol*. **40**, 446–452.

SCHMIDT, J. AND CALABRESE, R. L. (1992). Evidence that acetylcholine is an inhibitory transmitter of heart interneurones in the leech. *J. exp. Biol.* **171**, 329–347.

SIMON, T. W., OPDYKE, C. A. AND CALABRESE, R. L. (1992). Modulatory effects of FMRF-NH_2 on outward currents and oscillatory activity in heart interneurones of the medicinal leech. *J. Neurosci*. **12**, 525–537.

STRICKHOLM, A. (1981). Ionic permeability of K, Na and Cl in potassium-depolarized nerve. *J. Biophys.* **35**, 677–697.

SZCZUPAK, L., JORDAN, S. AND KRISTAN, W. B., JR (1993). Segment-specific modulation of the electrophysiological activity of leech Retzius neurones by acetylcholine. *J. exp. Biol.* **183**, 115–135.

THOMPSON, W. J. AND STENT, G. S. (1976). Neural control of heartbeat in the medicinal leech. II. Intersegmental coordination of heart motor neuron activity by heart interneurones. *J. comp. Physiol.* **111**, 281–307.

WANKE, E., CARBONE, E. AND TESTA, P. L. (1979). K^+ conductance modified by a titratable group accessible to protons from the intracellular side of the squid axon membrane. *J. Biophys*. **26**, 319–324.

WILSON, W. A., CLARK, M. T. AND PELLMAR, T. C. (1977). Tris buffer attenuates acetylcholine responses in *Aplysia* neurones. *Science* **196**, 440–441.

YAMAMOTO, D. AND SUZUKI, N. (1987). Blockage of chloride channels by Hepes buffer. *Proc. R. Soc. Lond. B* **230**, 93–100.

YAROWSKY, P. J. AND CARPENTER, D. O. (1978). A comparison of similar ionic responses to γ-aminobutyric acid and acetylcholine. *J. Neurophysiol.* **41**, 531–541.