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Chronotropic actions of Na^+,K^+,Cl^- cotransport inhibition in the isolated rat heart

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Abstract

The chronotropic actions of Na^+,K^+,Cl^- cotransport were investigated by studying the effects of the loop diuretics bumetanide and furosemide, specific inhibitors of the cotransporter, on an isolated rat sino-atrial node preparation. Application of bumetanide decreased the cycle length from 0.334 s (±0.087 S.D.) to 0.279 s (±0.083, $n = 16$, $P = 6.5 \times 10^{-6}$) in Hepes-buffered physiological salt solution (PSS). Similar decreases were recorded in bicarbonate-buffered PSS. Chloride channel blockers indicate that the tachycardia evoked by loop diuretics is not due their blocking of chloride channels. Thus, 4,4′-dinitrostilbene-2,2′-disulphonic acid (DNDS) and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) had a negative chronotropic action and 2-[(2-cyclopentyl-6,7-dichloro-2,3-dihydro-2-methyl-1-oxo-1*H*-inden-5-yl) oxy] acetic acid (IAA-94) produced no change in cycle length. Pharmacological manoeuvres indicate that the positive chronotropic action of loop diuretics is associated with catecholamine release. The positive chronotropic action of bumetanide was inhibited by the β-adrenoceptor antagonists, propranolol and atenolol, but was unaffected by atropine.

Keywords: Sino-atrial node, rat; Na^+,K^+,Cl^- cotransport; Loop diuretic; Intracardiac ganglion

1. Introduction

The coupled cation-anion Na^+,K^+,Cl^- cotransporter contributes to the regulation of intracellular ion concentrations. It utilizes the Na gradient to drive the uptake of K and Cl ions, and thus it seems likely in excitable cells it will modulate membrane potential. The rationale is that by acting as an inward chloride pump, cotransport makes $[Cl^-]_i$ closer to $[Cl^-]_o$, and has a depolarizing influence. We have tested this hypothesis in vascular smooth muscle and found it to be sustained; $[Cl^-]_i$ is maintained at a level above that predicted for a passive distribution and this is partly due to the functioning of Na^+,K^+,Cl^- cotransport (Davis et al., 1993). The steady-state $[Cl^-]_i$ in cardiac muscle has been examined in some detail and it is known that it is accumulated to values greater than expected on the basis of a passive electrochemical distribution (Vaughan-Jones, 1982). There is evidence to indicate that this is due to Na^+,K^+,Cl^- cotransport (Aiton et al., 1981; Liu et al., 1987; Van Kerhove and Vaughan-Jones, 1988).

In order to examine whether the regulation of membrane potential by Na^+,K^+,Cl^- cotransport might also occur in cardiac cells we investigated its contribution to the control of pacemaker activity in the isolated rat heart. The activity of the cotransporter was assayed by using the loop diuretics bumetanide and furosemide, specific inhibitors of the Na^+,K^+,Cl^- cotransport system (Chipperfield, 1986). However, bicarbonate-dependent chloride transport mechanisms are present in many excitable cells and may influence $[Cl^-]_i$ (Thomas, 1984). Moreover, anion channels may be permeable to HCO_3^- as well as Cl^-, and loop diuretics can inhibit processes other than cotransport (in this context chloride channels: see Cabantchik and Greger, 1992). Thus we examined the effect of Cl^--HCO_3^- exchange activation in the presence of HCO_3^- on the chronotropic activity of Na^+,K^+,Cl^- cotransport with a view to evaluating the physiological role of the cotransporter to cardiac function. Additionally, the actions of anion channel blockers were assessed to ascertain whether or not they mirrored the positive chronotropic actions of

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cotransport inhibitors. Preliminary reports of some aspects of this work have been published (Chipperfield et al., 1992b; Harper et al., 1992).

2. Materials and methods

2.1. Electrophysiological recording

Male Sprague-Dawley rats (120–420 g; Bantin and Kingman, UK) were used. The animals were killed by concussion and cervical dislocation and the heart quickly removed. The atria were separated and the area of the right atrium containing the sino-atrial node, bordering the ridge of the crista terminalis, was identified, isolated, and pinned out in a chamber (volume 0.5 ml), lined with Sylgard. The preparation was superfused (1–1.5 ml/min) with either a Hepes-buffered or bicarbonate-buffered physiological salt solution (PSS) in the range 36–38°C, but with a maximum deviation of 0.5°C in any individual experiment. The temperature of the experimental chamber and superfusing solutions was controlled by a Peltier thermoelectric element (Cambion, USA) and monitored by a separate thermistor probe in the chamber (Yellow Springs Instruments, USA). The composition of the Hepes-buffered PSS was in mM: NaCl, 140; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 2 glucose, 10; Hepes, 5; pH 7.4 at 20°C; 7.2 at 37°C; gassed with O_2; the bicarbonate-buffered PSS had a similar composition but with NaCl, 122; $NaHCO_3$, 25; gassed with 95% O_2/5% CO_2 to give a pH between 7.3 and 7.5.

Standard glass microelectrode techniques were used. Microelectrodes were pulled from 1.5 mm o.d. borosilicate glass containing a filament (Clark Electromedical, UK) and were filled with 0.5 M K acetate to preclude direct alteration of $[Cl^-]_i$ by leakage from the microelectrode (Thomas, 1978) and had DC resistances > 40 MΩ. The microelectrode was connected to an electrometer (Axoclamp 2A, USA) by an Ag-AgCl pellet with a second pellet in the recording chamber providing the earth reference. Electrophysiological signals were displayed on an oscilloscope and simultaneously fed to an electronic ratemeter (Neurolog 273, UK) the output of which was read out onto a chart recorder. Membrane potentials were also recorded on videotape through a modified Sony PCM 701. Taped signals were subsequently transferred to a microcomputer by means of a Cambridge Electronic Design (CED) 1401 interface and records were analysed using Spike 2 software (CED, UK).

Pacemaker action potential configuration was determined by recording from sino-atrial node preparations in which the impalement was stable throughout the recording period (since this necessitated intervals > 1.5 h, the number of experiments satisfying this criterion is correspondingly small). Five consecutive cycles were sampled in each condition and the mean parameters used in subsequent analysis. In some experiments, it was not possible to achieve extended recordings from a single cell, because of mechanical activity. Whereas reinserting the microelectrode was found not to affect the discharge activity, action potential shape usually was. In these situations spontaneous discharge was measured over a period of at least 30 s and used to calculate the cycle length (s) and frequency/min.

2.2. General characteristics

Data are presented from isolated sino-atrial node preparations in which the spontaneous action potential discharge was stable. Data from any preparations which displayed irregular spontaneous discharge, fluctuating frequency or in which the cycle length altered by more than 0.07 s in 30 min were rejected. Preparations could regularly be kept in good condition, as manifested by the above characteristics, for periods of up to 5 h.

2.3. Reagents

Pharmacological agents: atenolol, atropine, bumetanide, and propranolol were purchased from Sigma, UK and furosemide was a gift from Hoechst UK, UK. Chloride channel blockers: the dilsulphonic stilbene 4,4′-dinitrostilbene-2,2′-disulphonic acid (DNDS) and indanyl alkanoic acid 2-[(2-cyclopentyl-6,7-dichloro-2,3-dihydro-2-methyl-1-oxo-1*H*-inden-5-yl)oxy] acetic acid (IAA-94) were obtained from Pfaltz and Bauer, USA and Seamat, UK respectively; the anthranalic acid 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) was a gift from Dr R. Greger (Freiburg, Germany). All other reagents were of Analar grade from BDH, UK.

2.4. Statistical analysis

Data are presented as the means ± S.D. and were compared using Student's paired *t*-test (Statgraphics, USA).

3. Results

3.1. Effect of inhibition of Na^+,K^+,Cl^- cotransport by loop diuretics

The loop diuretic bumetanide is a specific inhibitor of the Na^+,K^+,Cl^- cotransport system (Chipperfield, 1986). Application of this agent (10 μM) clearly increased the rate of discharge of the sino-atrial node (see Fig. 1, Table 1), this effect being fully reversible on washout. Similar results were obtained for

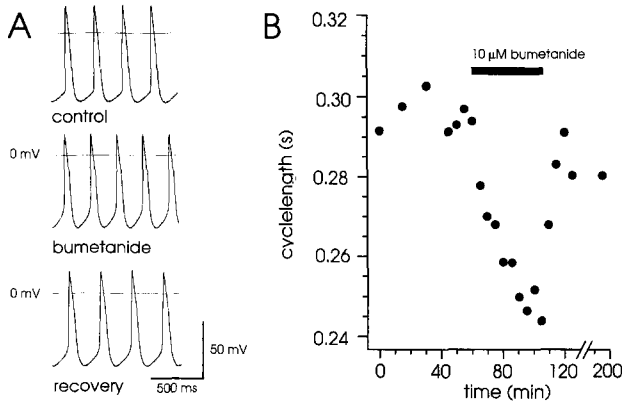

Fig. 1. A: The action of bumetanide on spontaneous action potentials recorded from an isolated sino-atrial node preparation, superfused with Hepes-buffered PSS, control; in the presence of 10 μM bumetanide, 30 min; recovery. B: Time course of development of the positive chronotropic action of bumetanide for this preparation.

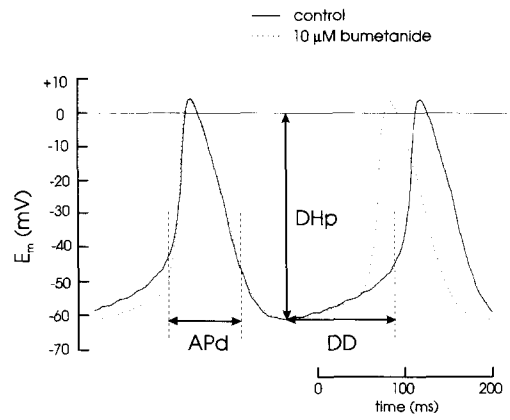

Fig. 2. Spontaneous action potential discharge recorded from the isolated rat sino-atrial node demonstrating the pacemaker parameters measured. The action potential duration (APd) was measured at the base i.e. at threshold level, the diastolic depolarization duration (DD) was measured from the peak diastolic hyperpolarization (DHp) to threshold. Action potentials were recorded in control, Hepes-buffered PSS solution and in the presence of 10 μM bumetanide, 30 min.

furosemide (100 μM) which decreased cycle length in the two preparations investigated from 0.323 and 0.290 s to 0.275 and 0.254 s respectively. The development of the positive chronotropic actions of the loop diuretics took approximately 20 min to attain steady state (this compares with a time constant of < 1 min to effect changes in $[Cl^-]_i$, E_m, and dilate α_1-adrenoceptor agonist-contracted arterial smooth muscle (Davis et al., 1991; unpublished observations).

Changes in pacemaker discharge may be due to changes in diastolic depolarization duration, amplitude, and threshold as well as action potential duration. Bumetanide did not alter the shape or duration of the action potential or the rate of the slow diastolic depolarisation and its positive chronotropic action is entirely due to decreasing the duration of the diastolic depolarization. An example of this is shown in Fig. 2. Bumetanide decreased the duration of the diastolic depolarization from 142 ms (±36) to 115 ms (±28, $n = 3$, $P = 0.050$). No significant differences in action potential overshoot duration, diastolic hyperpolarisation, or rate of slow diastolic depolarisations were recorded on application of bumetanide, but the threshold for slow depolarisation was shifted to more negative membrane potentials.

Table 1
The effect of 10 μM bumetanide on sino-atrial node discharge recorded whilst superfusing Hepes-and bicarbonate-buffered PSS

	Cycle length (s)	
	Control	Bumetanide
Hepes ($n = 16$)	0.334 ± 0.087 [189/min]	0.279 ± 0.083 [231/min]
Bicarbonate ($n = 6$)	0.241 ± 0.062 [249/min]	0.216 ± 0.051 [278/min]

[] AP discharge rate; mean values ± S.D. P values for t-tests: (a) Hepes-buffered, control:bumetanide, $P = 6.5 \times 10^{-6}$; (b) bicarbonate-buffered, control:bumetanide, $P = 0.0106$.

3.2. Relative contributions of Na^+,K^+,Cl^- cotransport and Cl^--HCO_3^- exchange to the regulation of pacemaker discharge

The experiments described above were performed in Hepes-buffered, nominally bicarbonate-free, PSS solution. This was to minimise, and isolate, any effect of Cl^--HCO_3^- exchange. These results raise the question of the presence of the positive chronotropic actions of the Na^+,K^+,Cl^- cotransporter in bicarbonate-buffered media and in order to determine whether cotransport activity has any significant effect upon sino-atrial rhythm its contribution must be tested at physiological levels of CO_2 and HCO_3^-. Bicarbonate-dependent chloride transport mechanisms are present in many excitable tissues, including cardiac (Vaughan-Jones, 1982), non-vascular smooth muscle cells (Aickin and Brading, 1990a,b) and neurones (Thomas, 1984), and may influence $[Cl^-]_i$. Recent work on smooth muscle has shown that the Cl^--HCO_3^- exchanger functions in conjunction with Na^+,K^+,Cl^- cotransport to increase $[Cl^-]_i$ above predicted equilibrium values, although their contributions can differ. Whereas in guinea-pig taenia coli cotransport is more important (Aickin and Brading, 1990b), in rat arterial smooth muscle there are approximately equal contributions (Davis, 1992). Furthermore, anion channels may be permeable to HCO_3^- as well as chloride (Bormann et al., 1987).

The contribution of Na^+,K^+,Cl^- cotransport to sino-atrial node discharge was assessed in the context of Cl^--HCO_3^- exchange activation by comparing the

Table 2
The chronotropic actions of chloride channel blockers

	Cycle length (s)	
	Control	Cl^- channel blocker
DNDS		
10 μM ($n = 3$)	0.353 ± 0.041	0.435 ± 0.062
100 μM ($n = 1$)	0.361	0.412
IAA-94		
10 μM ($n = 2$)	0.201, 0.360	No change
NPPB		
0.1 μM ($n = 2$)	0.237 *, 0.231	0.238 *, 0.235
40 μM ($n = 2$)	0.318, 0.301	0.352, 0.435

* Bicarbonate PSS. DNDS, 4,4′-dinitrostilbene-2,2′-disulphonic acid; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; IAA-94, 2-[(2-cyclopentyl-6,7-dichloro-2,3-dihydro-2-methyl-1-oxo-1*H*-inden-5-yl) oxy] acetic acid).

chronotropic actions of cotransport inhibition in Hepes-and bicarbonate-buffered PSS. The control cycle length was significantly less when superfusing with bicarbonate PSS, cycle length being 70% ($n = 6$) of that recorded in Hepes-buffered Ringer (see Table 1). This shift is likely to be due to the raised pH of the bicarbonate-buffered PSS (Satoh and Seyama, 1986). The positive chronotropic action of bumetanide was sustained in bicarbonate-buffered PSS (Table 1). The alterations induced by bumetanide in bicarbonate- and Hepes-buffered PSS solutions were similar.

3.3. The action of anion channel blockers

There is a body of evidence in the literature showing effects of the loop diuretic furosemide on Cl^- channels (Cabantchik and Greger, 1992) and although bumetanide particularly at the concentration employed here (10 μM) is more specific as an inhibitor of Na^+,K^+,Cl^- cotransport, it could act on other systems as well. To confirm the specificity of the action of bumetanide on the cotransporter, we used an anion channel blocker, the dinitrostilbene derivative, 2,2′-dinitrostilbene 4,4′-disulphonic acid (DNDS) reported to block cardiac chloride channels (Bahinski et al., 1989). This agent (10 μM, Hepes-buffered PSS) had a negative chronotropic action. In addition, superfusion with the chloride channel blocker indanyloxyacetic acid 94 (IAA-94), (Landry et al., 1987) 10 μM or NPPB (Cabantchik and Greger, 1992) 0.1 μM caused no change in cycle length (Table 2). However, NPPB at 40 μM had a negative chronotropic action (Fig. 3). Clearly, the effects of Cl-channel blockers are quite different from those of loop diuretics and it is thus improbable that loop diuretics are acting as Cl-channel blockers in this experimental situation.

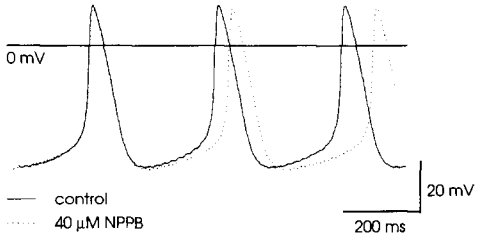

Fig. 3. Spontaneous action potentials recorded in control, Hepes-buffered, PSS solution and in the presence of 40 μM NPPB, 15 min.

3.4. The positive chronotropic action of loop diuretics is inhibited by β-adrenoceptor blockade

Pharmacologically 'denervating' the isolated sino-atrial node preparation by superfusing the muscarinic antagonist atropine at 10 μM (Adams and Nutter, 1992) and β-adrenoceptor antagonist propranolol at 10 μM (Harvey et al., 1990) increased the cycle length, in other words decreased the discharge rate from 0.318 s (± 0.051) to 0.621 s (± 0.272, $n = 4$). This demonstrates that there is a substantial amount of endogenous adrenergic transmitter release in this isolated, in vitro, preparation.

Subsequent addition of bumetanide to this superfusing solution caused no change. Withdrawal of propranolol and atropine but with bumetanide still present resulted in a rapidly developing decrease in cycle length. Whereas reapplication of atropine had no effect, propranolol inhibited the positive chronotropic action of bumetanide. An example of these manoeuvres is shown in Fig. 4. In two associated experiments bumetanide (10 μM) evoked decreases in cycle length to 77 and 83% of control values, addition of propranolol (10 μM) restored this to 109 and 110% of control values respectively. Correspondingly, the β_1-selective

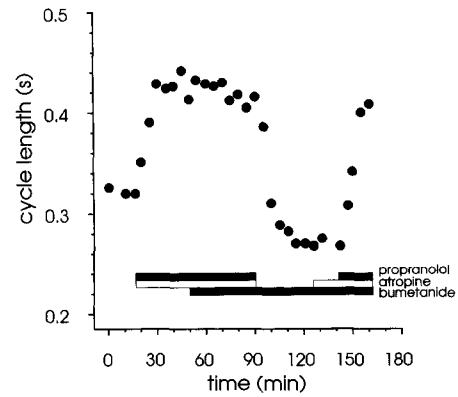

Fig. 4. The positive chronotropic action of bumetanide is blocked by the β-adrenoceptor antagonist propranolol (10 μM). Application of bumetanide in the presence of atropine (10 μM) and propranolol results in no alteration of discharge. Withdrawal of propranolol and atropine resulted in a rapidly developing tachycardia. Atropine had no inhibitory effect.

adrenoceptor antagonist atenolol reversed the bumetanide-induced change in cycle length (from 0.365 s (±0.157, $n = 3$), control; 0.309 s (±0.104), bumetanide (10 μM) to 0.372 s (±0.150), bumetanide and atenolol (10 μM)).

4. Discussion

The cycle length of spontaneous action potential discharge of the isolated sino-atrial node was affected by Na^+,K^+,Cl^- cotransport inhibition in a consistent way. The duration of the diastolic depolarization phase was reduced by a shift in the threshold to more negative potentials while the action potential duration and peak diastolic hyperpolarization were unaffected. The discharge of the sino-atrial node is a complex phenomenon. Decreases in the cycle length (increases in the discharge rate) of the sino-atrial node in these circumstances may be caused directly by changes in the characteristics of the currents contributing to the pacemaker discharge or indirectly by modulating the neural input to the sino-atrial node. Changes in diastolic interval could also be due to a positional shift of the pacemaker centre or a change in its size. Thus sympathetic nerve stimulation has been reported to produce shifts in the pacemaker region in guinea pig sino-atrial node (Choate et al., 1993). There are marked differences in the diastolic depolarization and its transition to the action potential between pacemaker and driven cells altering the diastolic interval and a pacemaker shift cannot be ruled out in these experiments.

The original rationale was that blocking cotransport with bumetanide would reduce chloride accumulation, hyperpolarize the cells of the sino-atrial node, and decrease pacemaker discharge. There is some evidence of cellular hyperpolarization of smooth muscle on treating the heart with Na^+,K^+,Cl^- cotransport inhibitors. Loop diuretics increased the coronary flow in the Langendorff perfused rat heart (bumetanide (10 μM) increased flow from 6.96 ml/min/g (±2.19) to 8.77 ml/min/g (±1.50, $n = 8$, $P = 2.8 \times 10^{-4}$) (unpublished observations)) as predicted from its hyperpolarizing and vasodilator actions on saphenous arterial smooth muscle (Davis et al., 1991; Chipperfield et al., 1992a). In the same series of experiments heart rate increased from 170 to 193 beats per min. However, the chronotropic action of this agent was attenuated by the time-dependent fall typically seen in such preparations, and was not taken to be statistically significant ($P = 0.058$).

That the tachycardia evoked by loop diuretics is not due to their action on chloride channels is confirmed by the effects of the putative chloride channel blockers DNDS, IAA-94 and NPPB which do not mimic the positive chronotropic effect of loop diuretics. The chronotropic effect of loop diuretics was preserved in the context of Cl^--HCO_3^- exchange activation, and accordingly such changes could well be of physiological significance.

Pharmacological manoeuvres indicate that the positive chronotropic action of loop diuretics is associated with catecholamine release, the action of bumetanide being unaffected by atropine but antagonised by β-adrenoceptor blockade.

Sympathetic nerve stimulation of the sino-atrial node produces a tachycardia comprised of two overlapping components with distinct diastolic depolarization characteristics (Choate et al., 1993) Neuropeptide Y (NPY) is co-localized with noradrenaline in cardiac sympathetic nerve endings and in vitro produces a slowly developing tachycardia (Lundberg et al., 1984) and voltage shifts in the activation of the pacemaker current I_f and $[Ca^{2+}]_i$ (see Chang et al., 1994).

Clear indications of a pacemaker discharge potentiation were apparent shortly after changing to the loop diuretic containing PSS; the maximum effect was around 20 min of superfusion. Here we report only the initial observations demonstrating the action of loop diuretics on pacemaker discharge. Clearly this is not simply the well-established effect of catecholamines on the diastolic depolarisation rate and the detailed mechanism of action requires further investigation.

The involvement of chloride ions in the regulation of cardiac performance has been enigmatic. Several reviews have described and considered the role of chloride channels in cardiac function (Ackerman and Clapham, 1993; Hume and Harvey, 1991). Recent descriptions suggest that a chloride current is involved in the autonomic regulation of cardiac cells (Harvey and Hume, 1989). β-Adrenoceptor-stimulated chloride currents were suppressed by muscarinic receptor stimulation. The β-adrenergic-operated chloride current was, however, found to be absent in sino-atrial node cells (Takano and Noma, 1992).

A minimal explanation for the present results is that, somehow, an inhibitory influence on catecholamine release is being removed by bumetanide. These results incline us to the view that loop diuretics are acting at a presynaptic site in the cardiac neuronal plexus. A direct test of this hypothesis would be to determine the chronotropic actions of bumetanide in tissue depleted of endogenous catecholamines. Persistent attempts to do just this, using 6-hydroxydopamine to discharge and prevent further synthesis of catecholamines, resulted in a reduction in pacemaker activity during superfusion of 6-hydroxydopamine followed by an increase on its removal (data not shown) but which was still susceptible to β-adrenoceptor blockade.

Ganglia have been identified on or in the atria of the heart. These have generally been regarded as vagal post-ganglionic cholinergic neurones but evidence is

now accumulating to indicate that the neurones in these ganglia also receive inputs from the efferent sympathetic nervous system and afferent cardiovascular mechanoreceptors within the heart (Ardell et al., 1981; Butler et al., 1990; Gagliardi et al., 1991). Furthermore, there is evidence for the presence of neurones forming functional afferent, efferent and local circuits within the cardiac plexus (Ardell et al., 1981; Moravec et al., 1986; Steele and Choate, 1994). As a result intracardiac neurones may have an integrative function and may well be capable of modulation of cardiac performance (Xu and Adams, 1993).

There is growing evidence that the cotransporter functions as an inwardly directed chloride pump to accumulate $[Cl^-]_i$ to values higher than predicted for a passive distribution in nerve cells, e.g. isolated rat superior cervical ganglion neurones (Ballanyi and Graffe, 1985). At the cellular level, inhibition of cotransporter operation should result in a decrease in $[Cl^-]_i$, membrane potential hyperpolarisation and decreased excitability. However, the overall effect on a neuronal network, even a simple one, cannot be predicted easily and requires to be distinguished by direct investigation of intracardiac ganglion neurones.

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