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# Modulation of Inwardly Rectifying $Na^+$-$K^+$ Channels by Serotonin and Cyclic Nucleotides in Salivary Gland Cells of the Leech, *Haementeria*

Werner A. Wuttke and Michael S. Berry

Biomedical and Physiological Research Group, School of Biological Sciences, University College of Swansea, Singleton Park, Swansea SA2 8PP, Wales

**Summary.** The electrically excitable salivary cells of the giant Amazon leech, *Haementeria*, display a time-dependent inward rectification. Under voltage clamp, hyperpolarizing steps to membrane potentials negative to about −70 mV were associated with the activation of a slow inward current ($I_h$) which showed no inactivation with time. The time course of activation of $I_h$ was described by a single-exponential function and was strongly voltage dependent. The activation curve of $I_h$ ranged from −72 to −118 mV, with half-activation occurring at −100 mV. Ion-substitution experiments indicated that $I_h$ is carried by both $Na^+$ and $K^+$ ions. 5-Hydroxytryptamine (5-HT) increased the amplitude of $I_h$ and its rate of activation. It also produced a positive shift of the activation curve of the conductance underlying $I_h$ ($G_h$) without altering the slope factor, thus indicating that the voltage dependence of $I_h$ was modulated by 5-HT. $Cs^+$ blocked both $I_h$ and the 5-HT-potentiated current in a voltage-independent manner, whereas $Ba^{2+}$ had little effect. It is concluded that 5-HT increases $I_h$ by modulating the inwardly rectifying $Na^+$-$K^+$ channels in the salivary cells. The effect of 5-HT may be mediated by an increase in adenylate cyclase activity since $I_h$ was increased by 8-bromo-cyclic AMP and by the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine. In contrast, $I_h$ was reduced by 8-bromo-cyclic GMP and by zaprinast (an inhibitor of cyclic GMP-sensitive phosphodiesterase). Cyclic GMP itself also reduced $I_h$, and the effect was specific to the 3′,5′ form; 2′,3′-cyclic GMP was inactive. The results suggest that the inward-rectifier channel may be modulated in opposite directions by cyclic AMP and cyclic GMP.

**Key Words** cyclic nucleotides · 5-HT · inward rectification · leech · salivary gland

## Introduction

Inward rectification is a membrane property that allows current to pass more readily into the cell than out and is usually reflected as a progressive increase in membrane conductance with increasing hyperpolarization. It has been described in a wide variety of cells such as skeletal muscle (Katz, 1949; Standen & Stanfield, 1980; Leech & Stanfield, 1981); heart muscle (DiFrancesco, 1981*a*,*b*; Vandenberg, 1987); smooth muscle (Benham et al., 1987; Edwards & Hirst, 1988); neurons (Benson & Levitan, 1983; Constanti & Galvan, 1983; Mayer & Westbrook, 1983; Crepel & Penit-Soria, 1986; Baker et al., 1987); photoreceptors (Hestrin, 1987); egg cells of marine invertebrates (Hagiwara, Miyazaki & Rosenthal, 1976; Hagiwara et al., 1978; Ohmori, 1978; Hagiwara & Yoshii, 1979) and blood cells (Kawa, 1989).

Inward-rectifier channels, like other voltage-dependent channels, may be modulated by neurotransmitters; for example, in cardiac muscle (Earm, Shimoni & Spindler, 1983), pituitary tumor cells (Bauer, Meyerhof & Schwarz, 1990), and in neurons of invertebrates (Benson & Levitan, 1983; Levitan & Levitan, 1988) and vertebrates (Stanfield, Nakajima & Yamaguchi, 1985; Mihara, North & Surprenant, 1987; Inoue, Nakajima & Nakajima, 1988; Yamaguchi et al., 1990). In contrast to the many studies that have been made on inward rectification itself, relatively little is known about its modulation, especially in cells other than nerve and muscle. For example, there is little data to compare effects on $K^+$-specific and nonspecific inward-rectifier channels. Also, it is not always clear whether the transmitter actually modulates the channel or activates a different type of channel with inward-rectifying properties. We extend these studies on modulation of inward rectification to salivary gland cells and show that 5-HT has a potentiating effect, probably mediated *via* cyclic AMP, while cyclic GMP is inhibitory.

Some of the results have been published in abstract form (Wuttke & Berry, 1991*b*)

## Materials and Methods

### Preparation

Specimens of the giant Amazon leech *H. ghilianii* were obtained from our breeding colony and maintained in freshwater aquaria

at 25°C. Experiments were performed on the anterior salivary glands which were dissected from the animal and secured to a layer of Sylgard® at the base of a Perspex experimental bath (volume 0.25 ml) by placing pins through the overlying endothelium. Physiological saline or other solutions (*see below*) flowed through the bath at a rate of about 10 bath volumes $min^{-1}$. Solutions could be changed rapidly without affecting recording conditions (Wuttke & Berry, 1988).

### Electrophysiology

Individual gland cells were voltage clamped with two bevelled, KCl-filled microelectrodes (10–30 MΩ) mounted on high speed steppers (Digitimer SCAT-02). An Axoclamp 2-A amplifier (Axon Instruments) was used, and a grounded shield was placed between the two electrodes to minimize capacitative coupling. The gain was normally set around 5000 V/V. Electrodes were positioned at an angle of 60° to each other to minimize spatial nonuniformities of membrane potential (Eisenberg & Engel, 1970), and their tips were separated by about one-third of the cell diameter. The largest cells (600–1000 μm) were chosen for study. They were generally held at, or close to, their resting membrane potential, and the effects of substances were examined on the currents evoked by hyperpolarizing voltage steps. The degree of space clamp of these giant cells is unknown, but the finding that the rate and voltage dependence of activation of the inward-rectifier current were smooth and continuous functions of the membrane potential suggests that sufficient clamp was obtained to avoid serious errors. In this respect the cells have the unusual advantage that they lack the intercellular coupling which is a property of most other exocrine gland cells (Wuttke & Berry, 1988).

A Ag-AgCl pellet in direct contact with the bath fluid was normally used as reference electrode. In some experiments with $Cl^-$-free solutions, however, a broken microelectrode ($\phi \sim 20$ μm) filled with 3 M KCl and connected to a KCl reservoir was used instead. The membrane potential was then measured differentially with respect to the free-flowing bath electrode which was placed downstream to the preparation, taking care to prevent any KCl contamination.

Signals were monitored on a storage oscilloscope (Tektronix 5111) and pen recorder (Brush 2200S) and stored on tape (Thorn EMI 3000 FM tape recorder).

### Solutions and Drugs

Physiological saline contained (in mM): NaCl 115, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, glucose 11, and Tris maleate 10 (pH 7.4). Alterations in $K^+$ concentration were compensated by changes in $Na^+$ concentration to maintain osmolarity. N-methyl-D-glucamine substituted for $Na^+$ in $Na^+$-free solution and also for $K^+$ and $Na^+$ in $K^+$-$Na^+$-free solution. Three different substitutes were used for $Cl^-$-free solution: (i) the appropriate salts of D-gluconic acid (containing 9 mM Ca-gluconate to compensate for the possible binding of $Ca^{2+}$ by gluconate (Kenyon & Gibbons, 1977)); (ii) K-gluconate and Na-methyl sulphate; (iii) K- and Na-sulphate (2 and 57 mM, respectively) also containing saccharose (76 mM). All solutions contained glucose (11 mM) and were buffered with 10 mM Tris maleate. The pH was adjusted to 7.4 with either NaOH or HCl ($Na^+$-free and $Na^+$-$K^+$-free solutions). The following substances (obtained from Sigma) were directly added to the solutions: 8-bromo-adenosine 3′,5′-cyclic monophosphate, 8-bromo-guanosine 3′,5′-cyclic monophosphate, guanosine 3′,5′- and guanosine 2′,3′-cyclic monophosphate (all sodium salts), 5-hydroxytryptamine creatinine sulphate (5-HT), 3-isobutyl-1-methylxanthine, CsCl and $BaCl_2$. Zaprinast (M&B22948, kindly supplied by Rhone-Poulenc Rorer) was prepared as a 5% stock solution in 1 N NaOH. Appropriate amounts were added to the saline, and the pH was readjusted to 7.4. Experiments were performed at room temperature (18–22°C).

### $K^+$-Selective Microelectrodes

Double-barrelled $K^+$-selective microelectrodes were made using a neutral carrier solution (Fluka, No. 60031) based on valinomycin (Oehme & Simon, 1976). The active barrel was silanized with a solution of 5% tributylchlorosilane (Fluka) in reagent grade carbon tetrachloride (BDH) as described by Borrelli et al. (1985). After the neutral carrier solution was introduced into the electrode tip the rest of the active barrel was backfilled with 0.5 M KCl. The reference barrel was filled with 3 M lithium acetate (pH 6.8; containing 10 mM LiCl) instead of 3 M KCl to avoid interference of KCl, leaking from the reference barrel, with the active barrel (Coles & Tsacopoulos, 1979). Membrane potentials measured with these reference barrels were similar to those measured with electrodes filled with 3 M KCl (Wuttke, 1990). The active and the reference barrels were connected by chlorided silver wires to the inputs of a varactor bridge amplifier (311J, Analog Devices; input resistance $10^{14}$ Ω) and the headstage of a Digitimer NL 102 amplifier, respectively. The potential of the reference barrel was subtracted electronically from the potential of the active barrel by a homemade differential amplifier to give the $K^+$ signal. Electrodes were calibrated in solutions of constant ionic strength, the 4-mM $K^+$ solution being identical to normal saline. In solutions with increased $K^+$ concentrations (10, 40, and 100 mM) NaCl was replaced by KCl in equimolar amounts. The slope of the electrodes (between 10 and 100 mM) was 51–54 mV. Ion concentrations were converted into activities by assuming an activity coefficient of 0.75 (Robinson & Stokes, 1959).

Results are presented as mean values ± SD.

## Results

### Time-Dependent Inward Rectification

#### *Current-Voltage Relationship*

When constant-current hyperpolarizing pulses of progressively larger amplitude are applied to the salivary cells of *Haementeria*, the resulting electrotonic potentials show an increasing sag indicative of inward rectification. This is clearly seen in the steady-state current-voltage relationship which also shows the presence of outward rectification for depolarizing pulses (Wuttke & Berry, 1988). In the present experiments under voltage clamp, salivary cells were held at, or close to, their resting level (−40 to −70 mV), which was positive to the range at which the hyperpolarizing electrotonic potentials sagged. Hyperpolarizing voltage steps to about −70 mV triggered first a capacitative current followed by a

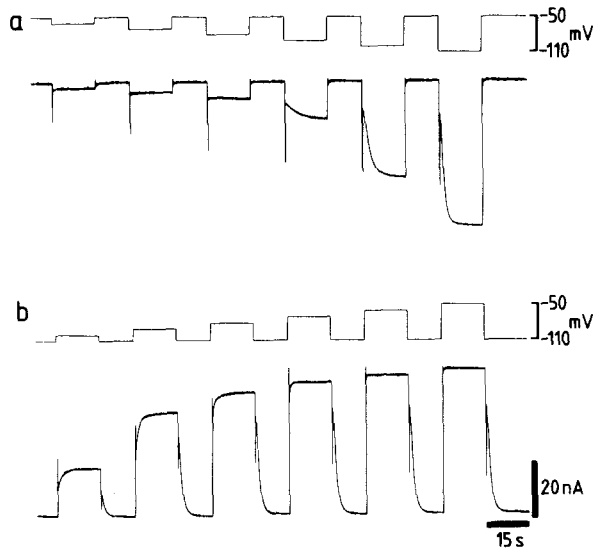


**Fig. 1.** (*a*) Hyperpolarizing voltage steps, progressively increasing by 10 mV from a holding potential of −50 mV, elicit a slow inward current ($I_h$) whose amplitude and rate of activation increase with increasing hyperpolarization. (*b*) Depolarizing steps, incrementing by 10 mV from a holding potential of −110 mV, produce slow outward relaxations, following the instantaneous currents, which reflect the deactivation of $I_h$. The chord conductances, calculated from the magnitude of the instantaneous currents, are 175 nS at −50 mV and 1000 nS at −110 mV.

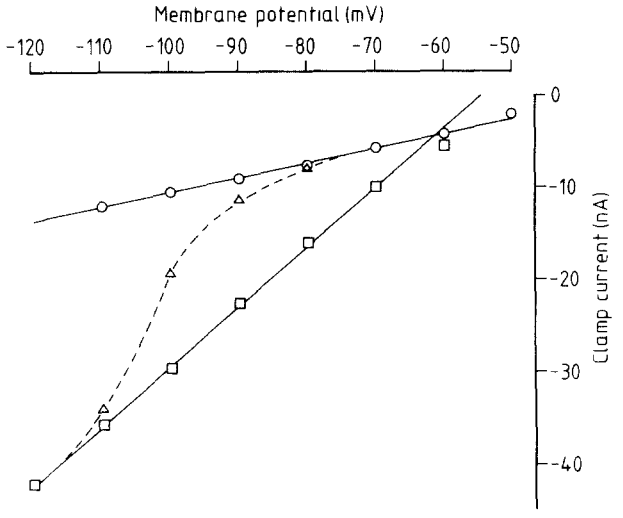


**Fig. 2.** Instantaneous (chord) current-voltage relationships upon stepping from −50 mV (circles) where $I_h$ was not activated and from −110 mV (squares) where $I_h$ was fully activated. The chord conductance plots are linear at both potentials, and their intersection gives an indication of the reversal potential of $I_h$ (ca. −60 mV). Triangles show plot of 'steady-state' current at the end of a 10-sec pulse against potential after stepping from a holding potential of −50 mV.

steady inward current whose development was too rapid to be resolved (Fig. 1). Larger hyperpolarizing steps evoked a larger instantaneous current followed by a slow inward relaxation whose time course and amplitude were dependent on the amplitude of the voltage step (Fig. 1*a*). The hyperpolarization-activated current did not inactivate during command pulses lasting up to 10 min. Upon repolarization to the holding potential there was a small inward tail current. The time course of deactivation was dependent on voltage (Fig. 1*b*).

The steady-state current-voltage relationship is plotted in Fig. 2 and shows an inwardly rectifying current starting to develop at a potential negative to about −70 mV. The instantaneous (chord) current-voltage relationship on stepping negative to the resting membrane potential was linear (Fig. 2).

The instantaneous current flowing at the beginning of a hyperpolarizing command was smaller than that flowing at the end, on repolarization to the holding potential, indicating an increase in membrane conductance associated with the inward relaxation. This suggests that the inward relaxation represents the activation of an inward current rather than the deactivation of outward current. For the cell in Fig. 1, the instantaneous (chord) membrane conductance was 175 nS at −50 mV and 1000 nS at −110 mV. In the following, the additional membrane conductance upon hyperpolarization from the resting membrane potential will be referred to as $G_h$ and the hyperpolarization-induced current as $I_h$.

## Ionic Basis of the Inward Rectifier

### *Reversal Potential of $I_h$*

Attempts were not made to determine the reversal potential by measurement of tail currents because it was considered that the speed of deactivation may cause a loss of part of the tail current in the capacitative transient and also because other currents were activated at the potential where reversal appeared to occur (Wuttke & Berry, 1991*a*).

The reversal potential was evaluated by determining the intersection of the instantaneous current-voltage relationships recorded at the resting membrane potential (where $G_h$ is not activated) and between −110 and −120 mV (where $G_h$ is strongly activated) (*see* Spain, Schwindt & Crill, 1987). The instantaneous current-voltage plots (Fig. 2) were linear in both cases, and the extrapolated lines intersected at a potential of $-60 \pm 4.4$ mV ($n = 9$). This is about 27 mV positive to the $K^+$ equilibrium potential ($E_K$) which was calculated from measurements of intracellular $K^+$ activity with $K^+$-selective

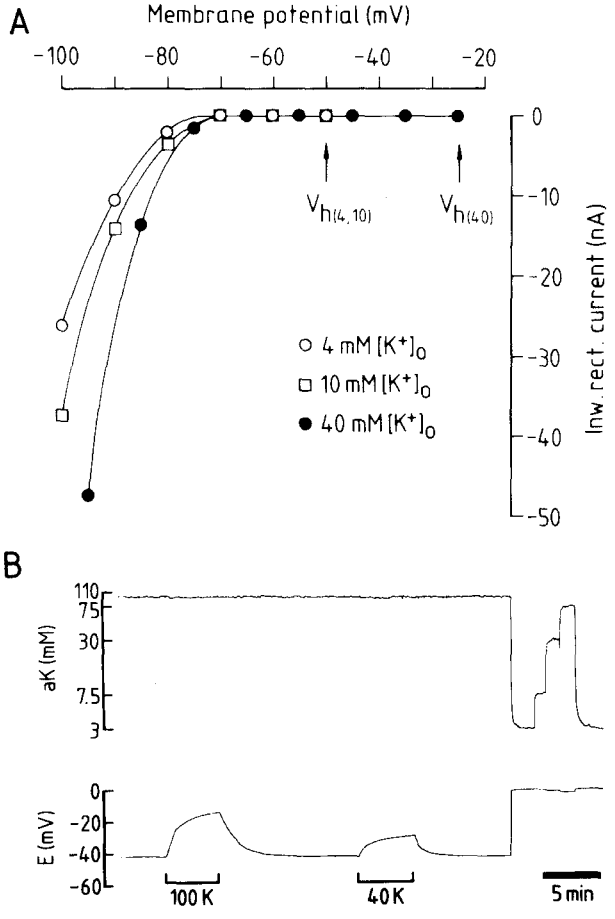


**Fig. 3.** (*A*) Effect of increasing $[K^+]_o$ upon hyperpolarization-activated currents. Current at the end of 12-sec hyperpolarizing steps from the holding potential ($V_h$, equal to the resting potential) is plotted against voltage. Open circles, control (4 mM $K^+$); squares, 10 mM $K^+$; filled circles, 40 mM $K^+$. $E_K$ changed from −87 (control) to −64 and −29 mV, respectively, with little change in the voltage at which $I_h$ became activated. (*B*) Increasing external $K^+$ concentration produces a depolarization without changing intracellular $K^+$ concentration. Upper trace is a recording of intracellular $K^+$ activity, measured with a double-barrelled $K^+$-selective microelectrode, and the lower trace is the membrane potential. At the end of the recording the microelectrode was removed from the cell and calibrated in solutions with different $K^+$ concentrations (i.e., 4, 10, 40 and 100 mM).

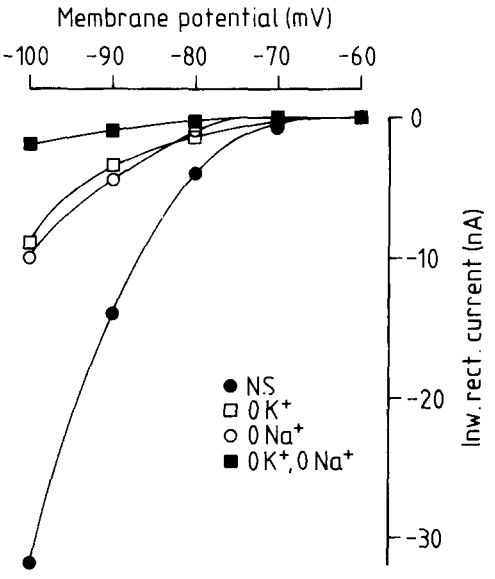


**Fig. 4.** Effects of removing $[Na^+]_o$ and $[K^+]_o$ on $I_h$. The plot shows the current-voltage relationship on stepping from a holding potential of −50 mV in normal saline (*NS*; filled circles) and in salines containing no $Na^+$ (open circles), no $K^+$ (open squares) and no $Na^+$ or $K^+$ (filled squares).

microelectrodes. The mean level of intracellular $K^+$ activity was 93.4 ± 9.6 mM ($n$ = 11), with $E_K$ equal to −87.2 ± 2.6 mV. The $Na^+$ equilibrium potential is known to be +45 mV (Wuttke & Berry, 1990).

### *Effect of Varying External $K^+$ and $Na^+$ Concentrations*

The value of the reversal potential of $I_h$ suggested that the rectifier is not $K^+$ specific. This was supported by the effect of raising the external $K^+$ concentration to 10 and 40 mM (Fig. 3*a*). There was an increase in $I_h$ and in slope conductance but little change in the potential at which the rectifier was activated. This lack of effect on activation threshold is a characteristic feature of mixed, $Na^+$-$K^+$ inward rectifiers and contrasts with $K^+$specific rectifiers, whose activation threshold depends on $[K^+]_o$.

$E_K$ was found to decrease from the mean value of −87.2 mV in normal saline to −64 and −29 mV, respectively, in saline containing 10 or 40 mM $K^+$. Intracellular $K^+$ was not changed by increasing external $K^+$ up to 100 mM (Fig. 3*B*) or by removal of external $K^+$ for 10 min (*not shown*). After about 5 min in $K^+$-free saline, the inward-rectifier current on stepping from the resting potential to between −100 and −130 mV was reduced to 42 ± 6.7% of control ($n$ = 15; Fig. 4).

The contribution of $Na^+$ to $I_h$ was examined by replacing $Na^+$ with N-methyl-D-glucamine which rapidly and reversibly reduced the amplitude of inward relaxations produced by hyperpolarizing commands (Fig. 4). On stepping from the resting potential to between −100 and −130 mV, $I_h$ was reduced to 45 ± 14% ($n$ = 7). Thus in this potential range $Na^+$ and $K^+$ ions appear to make an approximately equal contribution to $I_h$.

When both $Na^+$ and $K^+$ were omitted from the saline, there was a residual inward-rectifier current which was 9.2 ± 6.8% of control ($n$ = 5; Fig. 4). This may indicate an incomplete washout of these ions from the salivary gland or perhaps a contribution from other ions such as $Cl^-$.

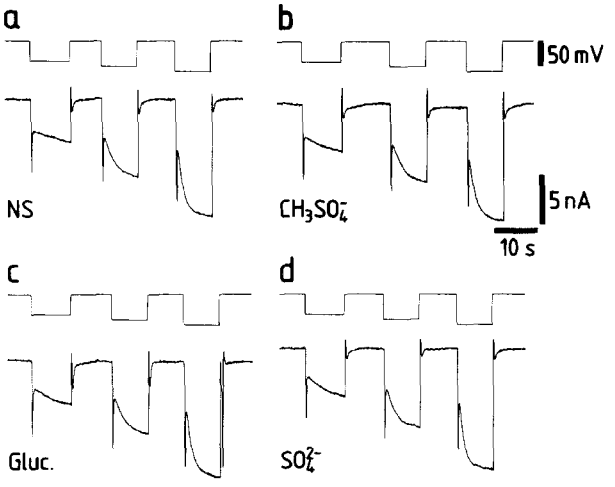


**Fig. 5.** Removal of external $Cl^-$ does not affect $I_h$. (*a*) Currents elicited by three, incrementing, hyperpolarizing voltage commands from a holding potential of −50 mV recorded in normal saline (*NS*). Pulse amplitudes were 40, 50 and 60 mV, respectively. (*b-d*) Same pulse protocol in the same cell following substitution of $[Cl^-]_o$ with methylsulphate ($CH_3SO_4^-$, *b*), gluconate (*Gluc.*, *c*), and sulphate ($SO_4^{2-}$, *d*). The apparent tail currents, which are potentiated in gluconate saline (*c*), probably represent subthreshold rebound responses produced by $Ca^{2+}$ influx (*see* Wuttke & Berry, 1991*a*).

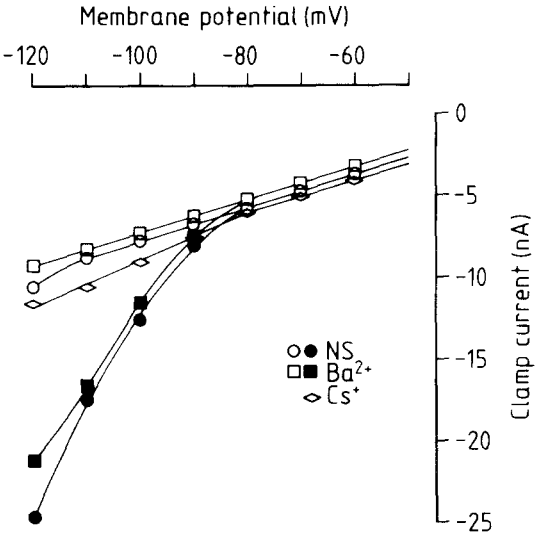


**Fig. 6.** $I_h$ is blocked by extracellular $Cs^+$ but not by $Ba^{2+}$. The current-voltage relationship is plotted for instantaneous currents (open symbols) and steady-state currents (filled symbols) produced by 10-sec hyperpolarizing steps from a holding potential of −40 mV. Circles represent control currents in normal saline (*NS*), squares are currents during 1 mM $Ba^{2+}$, and diamonds are currents during 2 mM $Cs^+$.

### *Effect of $Cl^-$ Substitution*

The salivary cells actively accumulate $Cl^-$ ions, which have a reversal potential of −23 mV (Wuttke & Berry, 1990); $Cl^-$ is therefore a possible contributor to $I_h$. Any contribution by $Cl^-$ would be expected to increase in $Cl^-$-free medium, but this was difficult to determine because the steepness of the activation curve for $I_h$ meant that an artifact of even a few millivolts produced a large change in measured rectifier current. After minimizing artifacts by use of a free-flowing 3-M KCl reference microelectrode, there was no change in $I_h$ (Fig. 5) or only a small change in either direction when external $Cl^-$ was removed. This was the same for three different substitutes, and any small changes could be accounted for by changes in the junction potential of the reference electrode. Chloride ions do not, therefore, appear to contribute to the inward-rectifier current.

### *Sensitivity of $I_h$ to $Cs^+$ and $Ba^{2+}$*

Hyperpolarization-activated cation currents carried by $Na^+$ and $K^+$ are characteristically blocked by low concentrations of external $Cs^+$ and are relatively insensitive to $Ba^{2+}$ (Halliwell & Adams, 1982; Mayer & Westbrook, 1983; Benham et al., 1987; Spain et al., 1987; McCormick & Pape, 1990*b*). This was also the case for the salivary cells of *Haementeria* (Fig. 6). Application of 0.1 mM $Cs^+$ reversibly reduced $I_h$ by 50–70%, with complete block at about 1 mM ($n$ = 11). In contrast, 1 mM $Ba^{2+}$ had little effect ($n$ = 4) and concentrations as high as 10 mM reduced $I_h$ by only 25–50% ($n$ = 3). $Cs^+$ or $Ba^{2+}$ had little effect on instantaneous components of the current responses. The block of $I_h$ by $Cs^+$ was not voltage dependent in the range tested (up to −120 mV); this contrasts with some other systems where the block increases with increasing voltage (Hagiwara et al., 1976; Gay & Stanfield, 1977; Mayer & Westbrook, 1983; Yamaguchi et al., 1990).

## Effect of 5-HT on $I_h$

### *5-HT Increases Amplitude and Rate of Activation of $I_h$*

Application of 5-HT enhanced $I_h$ with little change in instantaneous conductance at the beginning of the hyperpolarizing pulse (Fig. 7). For example, on stepping from the resting potential to between −100 and −110 mV, $I_h$ was increased to 214 ± 70% ($n$ = 25). This value was greater than the maximal amplitude of $I_h$ in the absence of 5-HT. The threshold concentration of 5-HT was about $10^{-8}$ M, with maximum potentiation occurring at $10^{-6}$ M (Fig. 8). Out-

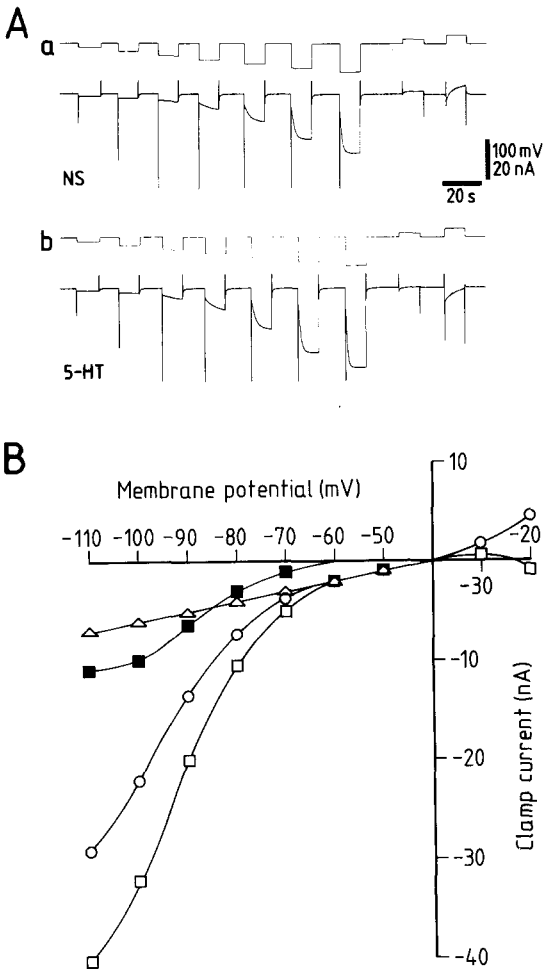


**Fig. 7.** 5-HT increases $I_h$. (*A*, *a*) Control currents in normal saline (*NS*) evoked by incrementing, hyperpolarizing voltage steps from a holding potential of −40 mV. The last two steps are in a depolarizing direction, and the first of these produces a small outward current while the second induces a larger outward current preceded by a transient inward current. (*A*, *b*) Same pulse protocol in the same cell during the presence of $10^{-5}$ M 5-HT. There is an increase in $I_h$, with little effect on instantaneous current. 5-HT abolishes the outward current produced by depolarizing steps, leading to an increase in net inward current (which now occurs in two phases). (*B*) Current-voltage relationships obtained for experiment in *A*. Instantaneous current (unchanged by 5-HT) is plotted as triangles; control steady-state currents are represented by circles; steady-state currents during 5-HT are open squares; 5-HT-induced currents (obtained by subtracting the *I-V* relationships before and after application of 5-HT) are shown by filled squares.

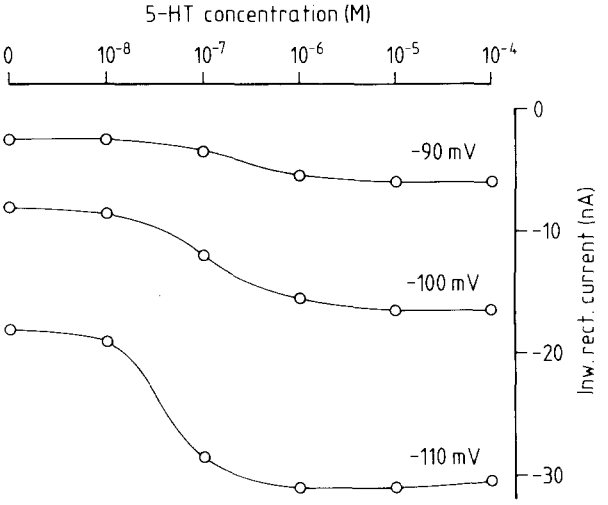


**Fig. 8.** Dose-response curves for the effect of 5-HT on $I_h$. The three plots show the values of $I_h$, on stepping from a holding potential of −50 to −90, −100 and −110 mV, respectively, against 5-HT concentration. In this cell the threshold concentration is about $10^{-8}$ M, with maximum effect at about $10^{-6}$ M. The currents are approximately doubled by 5-HT for each step.

ward (delayed) rectification was decreased by 5-HT (Fig. 7).

The time course of activation of $I_h$, following an initial short delay, could be described by a single-exponential function (Fig. 9*A*) and was strongly voltage dependent, with time constants ($\tau$) ranging from 3.62 ± 0.61 sec at −90 mV to 0.75 ± 0.16 sec at −120 mV (measurements on four cells; Fig. 9*B*). It can be seen from Fig. 9 that 5-HT increased the rate of activation of $I_h$ while maintaining the fit to a single exponential. Time constants decreased to 2.91 ± 0.7 (−90 mV) and 0.61 ± 0.1 (−120 mV).

Subtracting the inward-rectifier current during the action of 5-HT from the control current resulted in an *I-V* plot of the 5-HT-induced current (Fig. 7*B*). This exhibited a voltage dependence, with the response becoming progressively larger as the cell was hyperpolarized. The slope conductance then started to decrease as the inward rectifier approached maximal activation.

### *Effect of 5-HT on $I_h$ Activation Curve*

The steady-state activation curve of $I_h$ was constructed by plotting peak $I_h$ amplitudes, normalized to the maximal amplitude, against the membrane potential to which the cell was stepped during activation of $I_h$. The maximal amplitude was determined by first plotting chord conductance against voltage to find the voltage at which $G_h$ becomes maximal (Fig. 10*B*) and then measuring $I_h$ at this voltage ($I_{max}$). This method was checked by measuring tail currents elicited by repolarizing the membrane to holding level (−70 to −75 mV) following voltage steps of 15 sec to between −75 and −135 mV. This latter approach was not used initially because it seemed

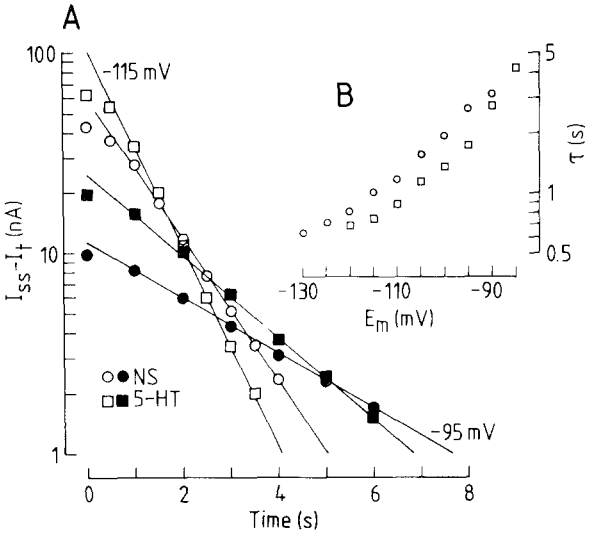


**Fig. 9.** Effect of potential and 5-HT on the activation kinetics of $I_h$. (*A*) Semilogarithmic plots of the inward current relaxations *versus* time induced by hyperpolarizing steps from −60 to −95 mV (filled symbols) and to −115 mV (open symbols). Circles represent control currents in normal saline (*NS*); squares are in the presence of $10^{-5}$ M 5-HT. Each point represents the difference between the steady-state inward-rectifier current, $I_{ss}$, and the inward-rectifier current at any given time after the onset of the voltage step, $I_t$. After an initial delay, the time courses of activation are each fitted to a single exponential, with time constants ($\tau$) of 2.67 and 1.01 sec for the control currents at −95 and −115 mV, respectively, and 1.73 and 0.74 sec, respectively, for the currents during 5-HT application. (*B*) Plot of $\tau$, determined as in *A*, against membrane potential in the presence (squares) and absence (circles) of $10^{-5}$ M 5-HT. Time constants are strongly voltage dependent and are decreased by 5-HT over the voltage range tested.

potentially unreliable: there was the possibility of contamination by other currents and the difficulty of losing part of the tail current in the capacitative transient. Nevertheless, essentially identical activation curves were obtained. For most of the voltage range the resulting data were well fitted by the Boltzmann equation:

$$I/I_{max} = (1 + \exp(V_m - V_{0.5}/s))^{-1}$$

where $V_m$ is the membrane potential, $V_{0.5}$ is the membrane potential at which $G_h$ is half activated, $I$ is the amplitude of the inward-rectifier current and $s$ is the slope factor which determines the steepness of the fitted curve (e.g., Hagiwara & Irisawa, 1989).

The activation range of $G_h$ was between −72 ± 5.4 and −118 ± 4.1 mV, with half-activation occurring at −99.9 ± 4.3 mV ($n$ = 12). The effect of 5-HT was a 5.0 ± 3.1 mV positive shift in the activation curve on the voltage axis without any change in the slope factor ($n$ = 5; Fig. 10). The voltage dependence of steady-state activation is quite steep, averaging 5.75 ± 0.4 mV ($n$ = 10). This slope factor corresponds to 4.4 gating charges moving across the entire membrane field. Due to the steepness of the activation curve, the 5-HT-induced shift would contribute, within a narrow potential range, to the increase in amplitude of $I_h$ by 5-HT as well as the decreased time constant of activation (*see* Discussion).

### *Effect of Extracellular $Cs^+$ and $Ba^{2+}$ on the 5-HT Response*

If the potentiation of $I_h$ by 5-HT is based upon modulation of the inward-rectifier channel, $Cs^+$ but not $Ba^{2+}$ should block this effect. Figure 11 shows that 1 mM $Cs^+$ completely eliminated inward rectification in the absence or presence of 5-HT, while 1 mM $Ba^{2+}$ had little effect (*not shown*). This, plus the results above, suggests that 5-HT increases $I_h$ by modulating a single type of channel rather than by activating an additional channel. Further information was gained by comparing the reversal potentials of control and 5-HT-enhanced $I_h$. In 5-HT the mean value of the reversal potential was found to be −60 ± 4.7 mV ($n$ = 8), which was identical to that of the control. This again favors the view that 5-HT has a modulatory action on the channel.

### *5-HT May Operate through Cyclic AMP*

Besides its action on inward rectification in the salivary cells, 5-HT produces a variety of other electrophysiological effects which are all mimicked by the membrane-permeable cyclic AMP analogue, 8-bromo-cyclic AMP (Wuttke & Berry, 1991*a*). In the present experiments, 0.5–1.0 mM 8-bromo-cyclic AMP reversibly increased the amplitude of $I_h$ to 216 ± 89% of control ($n$ = 5); it also increased the rate of activation of $I_h$ and shifted the activation curve in a positive direction (*see* Fig. 12). Three additional cells, however, were unaffected by 8-bromo-cyclic AMP, though they responded to 5-HT. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 0.2–1.0 mM) increased $I_h$ to 152 ± 26% of control ($n$ = 6) with little change in the instantaneous currents at the beginning of the voltage step (Fig. 13). These results strongly suggest that 5-HT mediates its effects *via* cyclic AMP.

### *$I_h$ Is Reduced by Cyclic GMP*

Some of the electrophysiological effects of cyclic AMP on the salivary cells are opposed by cyclic GMP (Wuttke & Berry, 1991*a*,*b*). It was of interest,

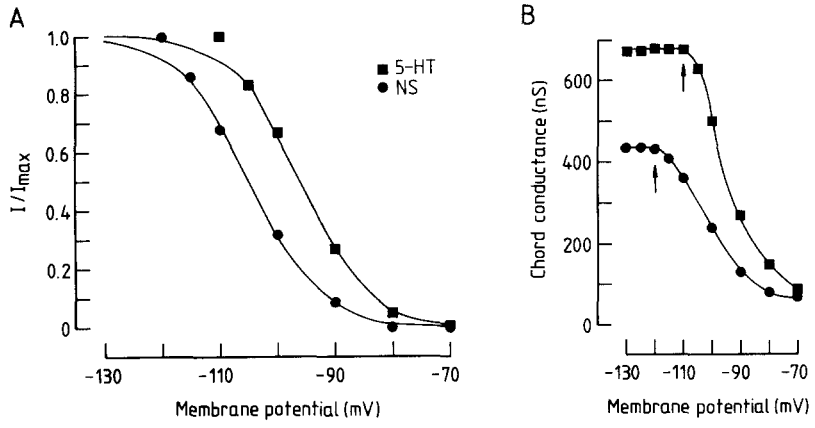


**Fig. 10.** (*A*) Effect of 5-HT on $I_h$ activation curve. The ordinate scale shows the amplitude of $I_h$, on stepping to various potentials from a holding potential of −60 mV, divided by the maximum value of $I_h$. This is plotted against the membrane potential during the step (circles). 5-HT shifted the activation curve in a positive direction (squares) by approximately 9 mV with no apparent change in slope. The lines are drawn according to the Boltzmann equation (*see* text), with a slope factor of 6 mV. Some deviation from a Boltzmann distribution can be seen at high membrane potentials. (*B*) A plot of chord conductance against membrane potential in the presence (squares) and absence (circles) of $10^{-5}$ M 5-HT. Arrows denote the membrane potential at which the conductance becomes maximal. Such plots were used to find $I_{max}$ (*see* text for details).

therefore, to determine the influence of cyclic GMP on inward rectification. In some systems cyclic GMP levels may be increased by sodium nitroprusside, which activates guanylate cyclase (Walman & Murad, 1987). Addition of $10^{-5}$–$10^{-4}$ M sodium nitroprusside to the salivary cells for 15 min produced little effect, either in the presence or absence of IBMX (*not shown*). However, 8-bromo cyclic GMP, and also cyclic GMP itself, reduced $I_h$. For example, cyclic GMP (1 mM) reversibly reduced the amplitude of $I_h$ to 62.5 ± 13% in 5–15 min ($n$ = 6) and reduced its rate of activation (Fig. 14). These actions were specific to the 3′,5′ form; 2′,3′-cyclic GMP had little or no effect. Furthermore, $5 \times 10^{-4}$ M zaprinast (an inhibitor of cyclic GMP-dependent phosphodiesterase) reduced $I_h$ to 89 ± 6% within 15–20 min; it was then washed out, but $I_h$ continued to decline to 58 ± 8% after 1 hr ($n$ = 3).

These results suggest that in the salivary cells of *Haementeria,* inward rectification may be modulated in opposite directions by cyclic AMP and cyclic GMP.

## Discussion

The electrically excitable salivary gland cells of *Haementeria* exhibit a hyperpolarization-activated, time-dependent cation current ($I_h$) which is potentiated by 5-HT and may be modulated (increased or decreased) by cyclic nucleotides.

### Ionic Basis of $I_h$

The current has the typical properties of mixed, $Na^+$-$K^+$ rectifiers which have been found in other cell types. For example, $I_h$ is activated slowly, it increases with increasing $[K^+]_o$ without a shift in the activation curve, and it is blocked by external $Cs^+$ but is resistant to $Ba^{2+}$. These features generally distinguish $Na^+$-$K^+$ rectifying currents from those carried by $K^+$ alone (DiFrancesco, 1981*a,b*; Halliwell & Adams, 1982; Earm et al., 1983; Mayer & Westbrook, 1983; Hille, 1984; Benham, et al., 1987; Hestrin, 1987; McCormick & Pape, 1990*a,b*; Tokimasu & Akasu, 1990).

The participation of other ions than $K^+$ is suggested by the reversal potential of $I_h$ (−60 mV) which is 27 mV positive to the $K^+$ equilibrium potential. An approximately equal contribution of $Na^+$ and $K^+$ ions to the current, determined in the voltage range −100 to −130 mV, is indicated by the similar reduction of $I_h$ (to 42–45% of control) by removal of external $Na^+$ or $K^+$. From the relative contributions of $Na^+$ and $K^+$ ions to the inward-rectifying current and the known values of $E_{Na}$ and $E_K$ (+45 and −87 mV, respectively) it was calculated that the permeability of the channels to $K^+$ is about 5.7 times that for $Na^+$. This figure was then used to calculate the reversal potential of $I_h$, where currents carried by $Na^+$ and $K^+$ are equal and opposite. Assuming that the permeability properties are independent of voltage, the value of the reversal potential was found to be −67 mV. This is in reasonable agreement with values estimated from instantaneous *I-V* relationships (Fig. 2).

The contribution of a $Cl^-$ conductance such as that described in *Aplysia* neurons (Chesnoy-Marchais, 1983) is unlikely since $I_h$ was almost completely blocked by removal of both external $Na^+$

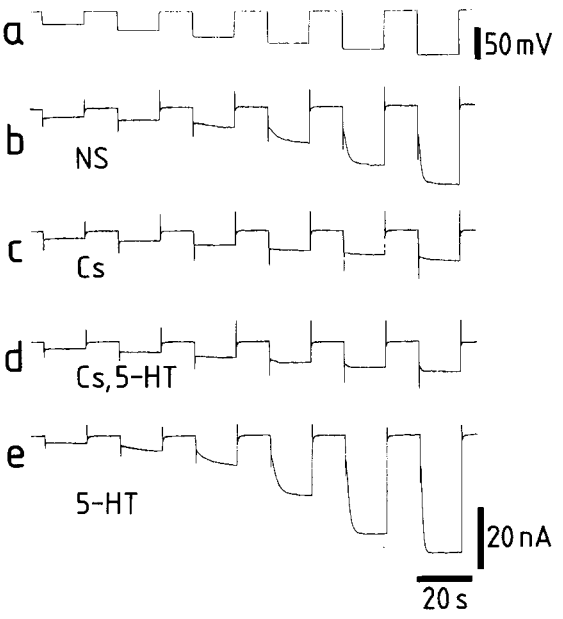


**Fig. 11.** $I_h$ and its potentiation by 5-HT are blocked by extracellular $Cs^+$ (1 mM). (*a*) Illustrates the pulse protocol for the experiments recorded in *b-e*. The cell was held at −50 mV and subjected to hyperpolarizing voltage steps which incremented by 10 mV up to −120 mV (the first step was 20 mV). (*b*) Control currents recorded in normal saline (*NS*). (*c*) $Cs^+$ blocks $I_h$, with little effect on the instantaneous currents. (*d*) In the presence of $Cs^+$, 5-HT ($10^{-5}$ M) has no effect. (*e*) On removal of $Cs^+$ $I_h$ is potentiated by 5-HT.

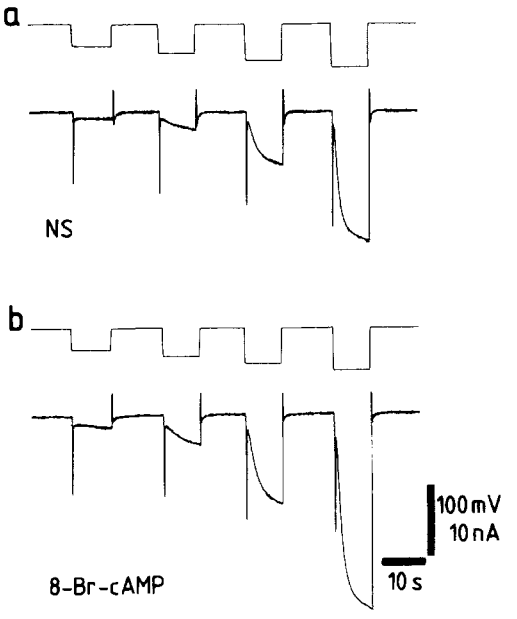


**Fig. 12.** Extracellular 8-bromo-cyclic AMP mimics the effect of 5-HT on $I_h$. (*a*) Control currents in normal saline (*NS*) evoked by incrementing, hyperpolarizing voltage steps from a holding potential of −50 to −110 mV. (*b*) In the presence of $5 \times 10^{-4}$ M 8-bromo-cyclic AMP (*8-Br-cAMP*) there is an increase in the amplitude and rate of activation of $I_h$, with little effect on the instantaneous current. In addition, the activation curve is shifted in a positive direction (*not shown*, but note the presence of $I_h$ during the first voltage step in *b* but not in *a*).

and $K^+$ and was unaffected in amplitude or kinetics by removal of external $Cl^-$. Chloride-free solution rapidly washes out extracellular $Cl^-$ but removes the ion very slowly from the cells (Wuttke & Berry, 1990) so that its lack of effect cannot be due to redistribution of $Cl^-$ across the membrane. None of the three $Cl^-$ substitutes produced a pharmacological block of $I_h$ which has been found in certain other cell types (Mayer & Westbrook, 1983; McCormick & Pape, 1990*a*,*b*).

### Potentiation of $I_h$ by 5-HT

The inward-rectifier current is increased in amplitude and rate of activation by 5-HT. These effects are presumably mediated *via* a specific receptor, in view of the low concentration of 5-HT required for an effect (threshold concentration $10^{-8}$ M). In this study we did not attempt to provide a pharmacological characterization of the 5-HT receptors (of which there may be a wide variety in invertebrates; e.g., Gerschenfeld & Paupardin-Tritsch, 1974).

It is not always easy to distinguish between a modulatory effect of a transmitter on a single type of channel and the activation of a different class of channel with similar inward-rectifying properties (e.g., Inoue et al., 1988). In the present experiments the evidence strongly favors the view that control and 5-HT-evoked currents are mediated by a single type of ion channel. For example: (i) The *I-V* relationship for $I_h$ is very similar to that for the 5-HT-induced current (obtained by subtracting *I-V* curves before and after 5-HT application). (ii) The rate of activation of the 5-HT-potentiated current retains a single-exponential relationship. (iii) The activation curve of $I_h$ is shifted by 2–9.5 mV on the voltage axis by 5-HT with no change in the slope factor. (iv) Low concentrations of extracellular $Cs^+$ abolish $I_h$ in parallel with blockade of responsiveness to 5-HT, whereas $Ba^{2+}$ has little effect on $I_h$ or the response to 5-HT. (v) There is no effect of 5-HT on the reversal potential of $I_h$, indicating similarity of ion selectivity of control and 5-HT-activated conductance channels.

The positive shift of the $I_h$ activation curve and increase in the rate of $I_h$ activation suggest that 5-HT enhances $I_h$ through an increase in the sensitivity of the underlying channels to the voltage difference across the membrane. This cannot be the only effect, however, because the inward-rectifier current in the

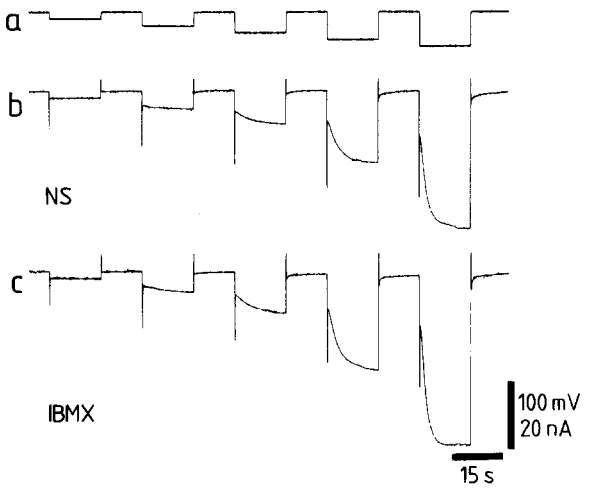


**Fig. 13.** 3-Isobutyl-1-methylxanthine (IBMX) potentiates $I_h$. (*a*) Pulse protocol: hyperpolarizing voltage steps, incrementing by 10 mV, were applied from a holding potential of −60 mV. (*b*) Control currents recorded in normal saline (*NS*). (*c*) Currents recorded 5 min after the introduction of $2 \times 10^{-4}$ M IBMX. The effects of $I_h$ are similar to those produced by 5-HT and 8-bromo-cyclic AMP.

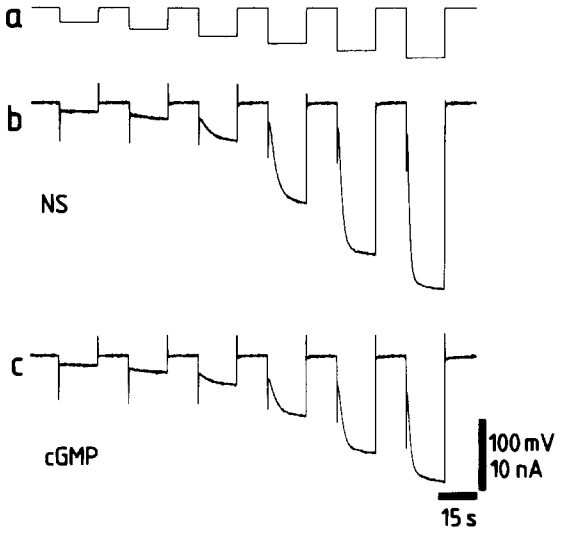


**Fig. 14.** Extracellular cyclic 3′,5′-guanosine monophosphate (cGMP) reduces $I_h$. (*a*) Pulse protocol: hyperpolarizing voltage steps, incrementing by 10 mV to −120 mV, were applied from a holding potential of −60 mV (first step was 20 mV). (*b*) Control currents in normal saline (*NS*). (*c*) Currents recorded in the presence of 1 mM cyclic GMP. There is a reduction in the amplitude and rate of activation of $I_h$.

presence of 5-HT was greater than its maximum value in the absence of 5-HT. Also, in some cells the shift was no more than 2 mV and seemed likely to have relatively little influence on $I_h$ in spite of the steep slope of the activation curve. The mechanism of the additional effect remains to be elucidated but presumably results from an increase in the number of functional channels and/or their probability of opening. If single-channel conductance was affected then there should be a change in the reversal potential, but none was found.

## Modulation of $I_h$ by Cyclic Nucleotides

The effects of 5-HT may be mediated by an increase in the concentration of cyclic AMP, which would regulate the availability of voltage-sensitive inward-rectifier channels. For example, reduction in phosphodiesterase activity or application of a membrane-permeable analogue of cyclic AMP both result in a marked enhancement of $I_h$. The involvement of a second messenger is also suggested by the long latency of the effect of 5-HT (about 2 min to reach maximum potentiation). Cyclic AMP appears to be involved in enhancement of inward-rectifier currents in other systems (Benson & Levitan, 1983; Bobker & Williams, 1989; Hagiwara & Irisawa, 1989; McCormick & Pape, 1990*a*; Tokimasa & Akasu, 1990), and it may also produce a reduction in current (Bauer et al., 1990). It is not universally involved, however (Mihara et al., 1987; Fargon, McNaughton & Sepulveda, 1990).

In *Haementeria* there is the likelihood that $I_h$ is modulated oppositely by cyclic AMP and cyclic GMP, since the latter produced a reversible reduction in $I_h$. This is further supported by the restriction of the effect to 3′,5′-cyclic GMP (the 2,′,3′ form is inactive) and by the inhibitory effect of zaprinast on the inward-rectifier current (zaprinast is reported to be an inhibitor of cyclic GMP-dependent phosphodiesterase: Winquist et al., 1984; Paupardin-Tritsch, Hammond & Gerschenfeld, 1986). Further experiments are necessary to identify any receptor agonists which might regulate $I_h$ *via* a cyclic GMP-dependent mechanism.

An action of cyclic GMP was unexpected since it would seem unlikely to enter the cell in high enough concentrations to influence the current. This may indicate an extracellular (or membrane) effect, perhaps with no physiological significance, though it is noteworthy that cyclic GMP is released from certain cells (possibly as a means of terminating its intracellular effects; *see* Hamet, Pang & Tremblay, 1989) and is thus available for extracellular actions. However, the inward movement of cyclic GMP across cell membranes is poorly understood. For example, it is widely assumed that 8-bromo cyclic nucleotides cross the cell membrane by simple diffusion through the lipid bilayer, yet the introduction of the 8-bromo group does not increase the hydrophobicity of these

molecules very much. There may be additionally a membrane transporter for uptake of cyclic nucleotides (Ullrich et al., 1991). Since cyclic GMP operates at very low intracellular concentrations, it cannot be ruled out that a sufficient amount enters the cell, down the high concentration gradient, to modulate the inward rectifier.

### 5-HT and Cyclic Nucleotides Modulate Other Conductances in the Salivary Cells

Besides its influence on $I_h$, 5-HT reduces outwardly rectifying (delayed) $K^+$ current, and thus, potentiates net inward $Ca^{2+}$ current associated with the action potential (*see* Fig. 7). 5-HT also reduces resting $K^+$ conductance, resulting in a small, subthreshold depolarization (Wuttke & Berry, 1991*a*). In the present experiments under voltage clamp, 5-HT produced a small inward current at the resting potential, but this was usually too small to be clearly seen at the normal amplification employed for measuring $I_h$. The effects on $K^+$ permeability result in increased excitability, increase in the amplitude and duration of action potentials, and reduction of adaptation during maintained depolarization.

It is noteworthy that the 8-bromo derivative of cyclic AMP mimics all the effects of 5-HT on the gland cells (Wuttke & Berry, 1991*a*), supporting the view that 5-HT acts *via* this cyclic nucleotide. Cyclic GMP also has effects on other channels in the salivary cells; for example, it reduces the duration and amplitude of action potentials, resulting eventually in a complete block (Wuttke & Berry, 1991*a*).

The salivary cells of *Haementeria* thus exhibit a variety of voltage-gated, transmitter-modulated conductances which are generally characteristic of nerve or muscle cells rather than those of exocrine glands. They may provide a useful preparation for the study of channel modulation (particularly $Na^+$-$K^+$ inward-rectifier channels, which have received relatively little attention) and present the opportunity to investigate the integration of responses to more than one second-messenger system.

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