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Ionic Mechanism of Cholinergic Inhibition in Molluscan Neurons

Abstract. *Acetylcholine, the inhibitory transmitter to the so-called* H*-neurons of molluscs, produces its effect by increasing the permeability of the subsynaptic membrane to chloride ions. The change in permeability gives rise to a net influx of this anion, which hyperpolarizes the neuron. The presence of an outward pump of chloride ions is postulated to account for the required electrochemical gradient. The participation of potassium ions in this inhibitory phenomenon was not detected.*

The inhibitory postsynaptic potentials (IPSP's) recorded in the so-called *H*-neurons of molluscs are probably produced by a cholinergic mechanism (*1*) since the iontophoretic application of acetylcholine (ACh) on these neurons produces a transient hyperpolarization (the ACh-potential) which has a reversal potential (E_{ACh}) similar to that of the IPSP's (E_{ipsp}) (*2*). Moreover, both ACh-potentials and IPSP's are blocked by *d*-tubocurarine and atropine (*1*, *2*). Our experiments deal with the ionic mechanisms of the IPSP and the ACh-potential in molluscan *H*-neurons. We have found, at variance with other reports (*3*), that the cholinergic hyperpolarization of these easily recognizable cells is due only to a net influx of Cl^- into the cell, this influx resulting from a specific increase in the membrane's permeability to Cl^-.

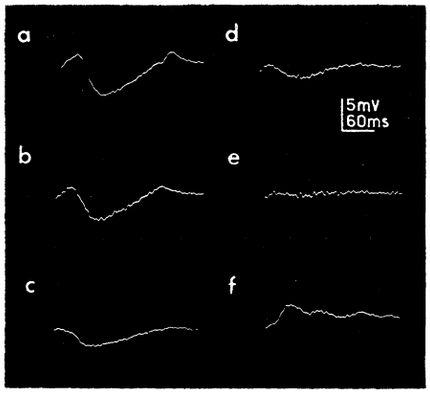

Fig. 1. Dependence on Cl^- of the cholinergic IPSP in an *H*-neuron. In (a) a control IPSP evoked by orthodromic stimulation ($[Cl]_o$ = 115 mmole/liter). The gradual reduction of $[Cl]_o$ to 80 (b), 60 (c), 40 (d), and 20 mmole/liter (e) produced a diminution of the IPSP amplitude. When the Cl-free solution bathed the preparation, the IPSP reversed (f). In all cases E_m equaled –44 mv.

Perioesophagic ganglia of the land snail *Cryptomphallus aspersa* were isolated in saline solution (*4*), and the *H*-neurons were impaled with double-barreled microelectrodes filled with 0.6*M* Na_2SO_4. One of the barrels was used for intracellular recording, the other for applying current to displace the membrane potential (E_m) to desired levels. Acetylcholine was delivered iontophoretically (*5*) from a micropipette filled with 1*M* acetylcholine iodide onto the neuronal cell bodies, which are devoid of synapses. Braking currents were used to prevent drug leakage and possible desensitization of the receptor (*6*). We changed the external concentrations of Cl^- and K^+ to investigate the effects of the IPSP's and the ACh-potential. We lowered the normal concentration of Cl ($[Cl]_o$) in the Ringer's solution (115 mmole/liter) stepwise by replacing Cl^- with SO_4^{2-} without changing the Na concentration; we kept the osmolarity constant by adding sucrose. The concentration of K^+ ($[K]_o$) in the saline was raised from the normal concentration of 4.9 mmole/liter to 15 mmole/liter by the addition of KCl. After every change in the solution the preparation was thoroughly washed with normal saline.

Figures 1 and 2 illustrate in two different *H*-neurons the effects of a stepwise reduction of $[Cl]_o$ on the IPSP's and on the ACh-potential. The control IPSP (Fig. 1a) gradually decreased in amplitude when $[Cl]_o$ was lowered to 80 (Fig. 1b), 60 (Fig. 1c), 40 (Fig. 1d), and 20 mmole/liter (Fig. 1e). A similar change in the amplitude of the ACh-potential was observed when $[Cl]_o$ was decreased (Fig. 2, b and c). The polarities of both the IPSP's and the ACh-potential reversed in a solution free of Cl^- (Fig. 1f and Fig 2d). These effects clearly indicate a participation of Cl^- in cholinergic synaptic inhibition. We obtained additional evidence for the role of Cl^- by changing the Cl^- concentration gradient across the membrane of *H*-neurons by an intracellular injection of NaCl. We did this by allowing current of appropriate polarity to flow between the two barrels of an intracellular twin microelectrode (*7*), one barrel being filled with KCl and the other with Na_2SO_4. Both the ACh-potential and the IPSP became reversed after currents of 50 to 100 na were passed for 60 to 90 seconds. The ACh-potential recovered its initial polarity and amplitude about 90 minutes after the injection was made.

When $[K]_o$ was increased to 15 mmole/liter, the amplitudes of both IPSP and ACh-potential were reduced. This effect might have been caused by either the change in the K gradient across the neuronal membrane or by the reduction of the membrane resistance of molluscan neurons by high $[K]_o$ (*8*, *9*). We could distinguish these two possibilities by investigating the effects of changes in $[K]_o$ on E_{ACh}. The E_{ACh} was obtained by displacing the membrane potential with inward current passed through one of the barrels of the intracellular microelectrode until the E_m at which the ACh-potential reversal was found. In many experiments the amplitude of the ACh-potential was

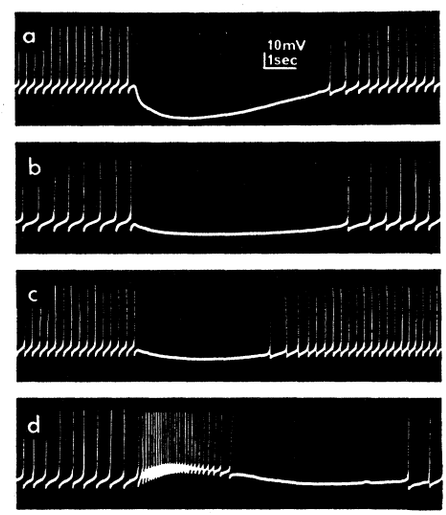

Fig. 2. Effect of changes in $[Cl]_o$ on the ACh-potential in an *H*-neuron. The E_{ACh} was previously measured and found to be –65 mv. (a) Injection of ACh when the cell is bathed in a Ringer solution containing 115 mmole of Cl^- per liter. In (b) and (c) the amplitude of the ACh potential is reduced when $[Cl]_o$ is lowered to 60 and 40 mmole/liter respectively. In (d) the ACh-potential reversed its polarity when the entire Cl^- content of the bathing solution was removed. The initial resting potential of the *H*-neuron [–44 mv in (a)] was slightly modified when some other records were taken, being –46 mv in (b), –44 mv again in (c) and –46 mv in (d).

Table 1. Effects of changes in $[Cl]_o$ and $[K]_o$ on E_{ACh} (millivolts) in individual experiments. The numbers given in parentheses are the means ± the standard errors. The mean change between the E_{ACh}'s in the 15 m*M* K solution and those in the control was -1.39 ± 0.74 ($P > .05$). The mean change between the E_{ACh}'s in the Cl-free solution and those in the control was -51.5 ± 5.6 ($P < .001$).

Saline solution		
15 m*M* K	Control	Cl-free
−53.0	−58.0	
−53.0	−53.0	
−55.0	−55.0	
−55.0	−56.0	
−60.0	−60.0	
−54.0	−54.0	
−51.5	−57.0	−26.0
−60.0	−60.0	3.0
−60.0	−61.0	3.0
	−46.0	− 8.0
	−51.0	−10.0
	−68.0	0
	−52.5	3.0
(−55.7 ± 1.1)	(−56.2 ± 1.5)	(−5.0 ± 4.1)

plotted against E_m, and E_{ACh} was obtained by interpolation. It was observed in most neurons that, when E_{ACh} was repeatedly measured in the same cell, the values varied by as much as 5 mv. These fluctuations were considerably greater than those we observed when measuring the E_{ipsp} of molluscan neurons that present a noncholinergic inhibition (*9*). A mean E_{ACh} value of -56.2 ± 1.5 mv was thus obtained in 13 *H*-neurons (see Table 1).

The replacement of the bathing solution by a solution free of chloride ions led to marked changes in E_{ACh}. In Table 1 the values recorded in the control solution are given in the central column, and those obtained in Cl-free saline are in the right column. The mean E_{ACh} value was reduced from −56.2 in normal saline to -5.0 ± 4.1 mv in Cl-free saline, the change being statistically significant ($P < .001$).

An increase in $[K]_o$ from 4.9 to 15 mmole/liter did not produce a significant change in E_{ACh}. While E_{ACh} was slightly reduced in high $[K]_o$ in some neurons (left column), the mean decrease of -1.39 ± 0.74 mv is not statistically significant ($P > .05$).

These results indicate that in *H*-neurons ACh produces an increase only in the membrane permeability to Cl^-. A similar ionic mechanism is responsible for synaptic inhibition in other invertebrates (*10*). The influx of Cl^- produced by ACh indicates that this anion is not distributed in electrochemical equilibrium with $[Cl]_o$ and E_m. This may be explained by the existence of a pump that extrudes Cl^- from the cell, making $[Cl]_i$ lower than would be expected from a passive distribution. The outward Cl^- pump would make the equilibrium potential for Cl (E_{Cl}) more negative than E_m.

The ionic basis of cholinergic inhibition in the land snail *Helix aspersa* has been previously examined by Kerkut and Thomas (*3*), who reported that changes in both Cl^- and K^+ permeability were involved. There are three major differences between these results and ours.

1) These authors studied the effect on E_{ACh} of a simultaneous fourfold decrease in $[Cl]_o$ and a fourfold increase in $[K]_o$; E_{ACh} was reduced by 30 mv. When $[Cl]_o$ alone was reduced by a factor of four, E_{ACh} fell −27 mv. These authors therefore concluded that the change in Cl^- permeability accounted for 90 percent of the synaptic current, while the change in K^+ permeability accounted for 10 percent. Kerkut and Thomas did not discuss the fact that the values of E_{ACh} they obtained in the same cell varied by as much as 4.5 mv. These spontaneous variations in E_{ACh} were larger than the effect of raising $[K]_o$. No statistical analysis of their findings was given.

2) An important difference between the results obtained in *Helix aspersa* and those we report concerns the values of E_{ACh}. From the results of Kerkut and Thomas one can calculate a mean E_{ACh} of −94 mv in a series of 12 neurons, while the average E_{ACh} measured by us is near −56 mv. If both the IPSP's and ACh-potential are due to the diffusion of Cl^- into the cell, this relation may be expressed by the following equation:

$$E_{ipsp} \text{ or } E_{ACh} = E_{Cl} = -RT/F \ln ([Cl]_o/[Cl]_i) \quad (1)$$

where R is the gas constant; T, the absolute temperature, and F, the faraday. Thus, it is possible to calculate the expected E_{ACh} provided $[Cl]_i$ is known. Recently, Kerkut and Meech (*13*), using a Cl-sensitive microelectrode, found in *Helix* a $[Cl]_i$ of 8.7 ± 0.4 mmole/liter in *H*-neurons. Since in the experiments of Kerkut and Thomas $[Cl]_o$ was 109 mmole/liter and $[Cl]_i$ was 8.7 mmole/liter the expected E_{ACh} would be −64 mv, that is, some 30 mv less negative than the E_{ACh} recorded by these authors. From our results, a $[Cl]_i$ of about 12 mmole/liter can be calculated from Eq. 1, a value not far from that determined by Kerkut and Meech (*11*).

3) A final point concerns the identity between E_{ipsp} and E_{ACh} which in *Aplysia* was considered as an important argument in favor of the transmitter role of ACh (*2*). This identity was not found in *Helix aspersa* where it was reported that E_{ACh} is 10 to 20 mv less negative than E_{ipsp} (*3*). In our experiments we found a very close agreement between E_{ipsp} and E_{ACh} (Table 2); in nine *H*-neurons the difference between E_{ipsp} and E_{ACh} is 0.65 ± 0.83 mv and is not statistically significant.

Table 2. Values of E_{ipsp} and E_{ACh} in *H*-neurons of *Cryptomphallus aspersa*. The numbers in parentheses are the means ± the standard error. The mean difference for paired data is -0.65 ± 0.83 ($P > .1$).

E_{ipsp} (mv)	E_{ACh} (mv)
−62.0	−63.0
−59.0	−60.0
−52.0	−53.0
−53.0	−52.5
−54.0	−53.0
−52.0	−50.0
−58.5	−60.0
−51.0	−57.0
−61.0	−61.0
−55.5	−55.0
(−55.8 ± 1.3)	(−56.5 ± 1.4)

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