Ionic Mechanisms of Cholinergic Excitation in Molluscan Neurons

Abstract. *Acetylcholine appears to be an excitatory transmitter at synapses on two different types of molluscan nerve cells: the so-called* D- *and* CILDA *neurons. The action of this substance is different in the two cases. In* D-*neurons, this compound increases the permeability of the subsynaptic or somatic membrane to chloride ions, and through a net efflux of this anion, depolarizes the cell. In* CILDA *neurons, on the other hand, acetylcholine depolarzies the cell by increasing its permeability to sodium ions.*

Pharmacological analysis of the nervous system of some molluscs (*1*) has demonstrated the presence of a group of cells, called *D*-neurons, characterized by an excitatory synaptic input which is probably cholinergic and by the absence of any inhibitory input. Iontophoretic application of acetylcholine (ACh) on the somatic membrane of these neurons produces a depolarization, the so-called ACh-potential. A similar response is observed in *CILDA* neurons (*2*), which also have a cholinergic excitatory synaptic input as well as a noncholinergic excitatory synaptic input and a long-lasting type of inhibition (*2, 3*).

We have studied the ionic basis of the cholinergic excitatory action in both types of neurons and have found that the mechanism by which ACh depolarizes is different in the two cases, causing a net efflux of Cl^- in *D*-neurons and a net influx of Na^+ in *CILDA* neurons.

Intracellular recordings from the neurons of the land snail *Cryptomphallus aspersa* were made with double-barreled microelectrodes filled with 0.6*M* K_2SO_4 as previously described (*4*). Injections of ACh were performed as described in our paper on *H*-neurons (*5*).

The reversal potential of the ACh-potential (E_{ACh}) was measured in *D*-neurons as described for *H*-neurons (*5*). In the case of *CILDA* cells, this technique could not be applied since the application of strong currents damaged the neurons in many cases. For this reason, the E_{ACh} was obtained by plotting the amplitudes of ACh-potentials against the membrane potential (E_m) at which they were recorded (usually between −80 and −30 mv). A straight line fitting these experimental points was drawn, in most of the cases by means of the least squares method; by extrapolating this line, E_{ACh}, the E_m value at which the ACh-potential amplitude is null, was obtained. The ACh injections were always separated by intervals of 60 to 90 seconds to avoid receptor desensitization effects (*6*). When the cells were bathed with solutions different from the normal saline (*7*), only four experimental points were obtained to minimize possible ionic shifts. The modified solutions were: (i) a Cl^--free solution in which SO_4^{2-} replaced the entire Cl^- content; (ii) a Cl^--free solution containing a low concentration of Na^+ (32 mmole of Na^+ per liter, 25 percent of the normal content); in both solutions the osmolarity was kept constant by the addition of sucrose; (iii) a solution containing an increased amount of K^+ (15 mmole/liter); and (iv) a solution containing an increased amount of Ca^{2+} (24 mmole/liter). The last two solutions were slightly hypertonic.

The effects of some of these solutions on the ACh-potential recorded in *D*-neurons are shown in Fig. 1. When a Cl^--free solution bathed the cell (Fig. 1b), the response to ACh recorded at the same E_m was nearly 30 percent greater than that of the control. The replacement of the control solution by the Cl^--free saline having a reduced amount of Na^+ (Fig. 1c) produced an effect similar to that of the Cl^--free Ringer solution. Afterward, the same *D*-neuron immersed in normal saline was depolarized to −20 mv when outward current was passed through the second barrel (Fig. 1a′). In this condition, the injection of ACh evoked a hyperpolarization. When the normal saline was replaced with a Cl^--free solution while the resting potential was kept at −20 mv (Fig. 1b′), the ACh-potential again reversed so that injection of ACh produced a depolarization.

Table 1 summarizes the value of E_{ACh} recorded in a series of *D*-neurons subjected to changes in their ionic environment. The mean control value of E_{ACh} (−25.1 mv) was reduced to −2.4

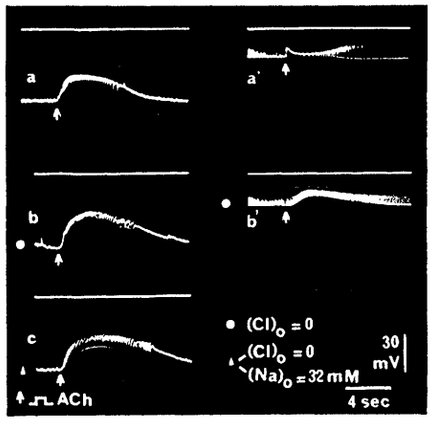

Fig. 1. Effect of changes in the ionic composition of the bathing solution on the ACh response of a *D*-neuron. The recordings were displayed in a double-beam cathode-ray oscilloscope. The upper trace corresponds to the zero potential level, and the lower trace displays the intracellular recording. Acetylcholine was applied iontophoretically (arrows) by passing currents of 100 na during 300 msec. (a) The ACh-potential recorded when the neuron was artificially hyperpolarized from –44 to –60 mv; control saline solution. (b) The neuron was immersed in a Cl^--free solution. Notice the increase in the amplitude of the ACh-potential. (c) The Na^+ content of the Cl^--free Ringer was lowered to 32 mmole/liter (25 percent of the normal content). Notice that the depolarizations in (b) and (c) are similar. (a′) The neuron was artificially depolarized to –20 mv. Application of ACh produced a hyperpolarizing response. (b′) The E_m is maintained at –20 mv, and the neuron is bathed in a Cl-free Ringer solution. Application of ACh produces a depolarization.

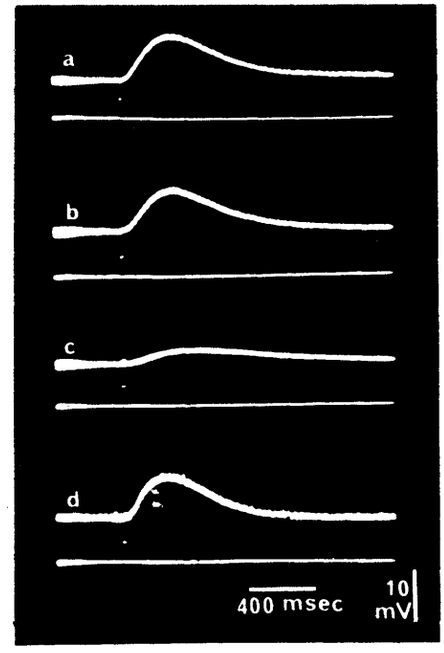

Fig. 2. Effect of changes in the ionic composition of the bathing solution on the ACh response of a *CILDA* neuron. Recording displaying in a double beam cathode-ray oscilloscope. Upper trace corresponds to intracellular recording. Lower trace monitors the ACh injecting current. (a) The E_m equals –58 mv. The bathing solution is normal saline. (b) Cl-free Ringer. (c) Cl-free Ringer containing only 25 percent of the normal Na^+ content (32 m*M*). Notice the lack of effect of changes of Cl^- and the decrease of ACh response when Na^+ is lowered. (d) The ACh-potential recovers its initial amplitude after the neuron has been washed with normal saline.

Table 1. Values of E_{ACh} (millivolts) in *D*-neurons in different ionic environments. The numbers in parentheses are the means ± the standard errors. The mean change (calculated for paired data) between the E_{ACh} in the control and chloride-free solutions was −22.8 ± 2.64 mv ($P < .001$); between those in the control and chloride-free, low Na^+ solutions, −24.0 ± 2.44 mv ($P < .001$); between those in the control and 24 m*M* Ca^+ solutions, 0 mv; between those in the control and 15 m*M* K^+ solutions, −0.75 ± 0.48 mv ($P > .1$); and between the E_{ACh}'s in the chloride-free and the chloride-free, low Na^+, 0.20 ± 0.20 mv ($P > .1$).

Solution				
Control	Cl-free	Low Na^+, Cl-free	24 m*M* Ca^+	15 m*M* K^+
−27.0	− 3.0	− 3.0		−27.0
−11.0	− 0.5		−11.0	
−34.0	−17.0	−18.0		
−21.0	7.0		−21.0	−20.0
−30.0	0	0	−29.0	−28.0
−26.0	2.0	− 2.0	−27.0	−26.0
−27.0	− 5.0	− 5.0		
(−25.1±2.79)				
	(−2.35±2.83)			
		(−4.80±3.60)		
			(−22.0±4.04)	
				(−25.3±1.80)

mv in the Cl^--free solution, but the Cl^--free solution with reduced Na^+ content had no further effect on the E_{ACh} of *D*-neurons, the mean value of −4.8 mv obtained in this condition being very close to that measured after the removal of Cl− alone (Table 1). The participation of a change in Ca^{2+} and K^+ permeabilities in the mechanism generating the ACh-potential can be ruled out, since the increase in the external concentrations of both cations had no effect on the E_{ACh} (Table 1). From the results presented one may conclude that only changes in the permeability to Cl^- are involved in the genesis of the ACh-potential in *D*-neurons.

A similar series of experiments was performed in the other type of cells, the *CILDA* neurons. Figure 2 illustrates an experiment in a *CILDA* neuron with a resting potential of −58 mv. The iontophoretic application of ACh onto the cell bathed with a normal solution provoked a depolarization of 10 mv (Fig. 2a). The removal of Cl^- from the cell environment did not affect the amplitude of the ACh-potential (Fig. 2b). On the other hand, when the external concentration of Na^+ was lowered to 32 mmole/liter, the amplitude of the response markedly decreased (Fig. 2c). This effect was rapidly reversed when the preparation was washed with the control saline (Fig. 2d). The application of tetrodotoxin 10^{-7} g/ml had no effect on the amplitude of the ACh-potential.

In Table 2 the effect produced on E_{ACh} by changes in the Cl^-, Na^+, Ca^{2+}, and K^+ concentrations is summarized for a series of *CILDA* neurons. The mean E_{ACh} in normal saline was near 0 mv. When the external concentration of Na^+ was lowered to 32 mmole/liter the mean E_{ACh} was increased by 20 mv. The removal of Cl^- or the increase in the external concentration of K^+ and Ca^{2+} did not affect E_{ACh} of the *CILDA* neurons. These results indicate that ACh caused in *CILDA* neurons an increase of their membrane permeability to Na^+.

The results on *D*-neurons confirm evidence previously obtained by Frank and Tauc in *Aplysia* and by Oomura *et al.* in *Onchidium* (*8*), and they eliminate the possibility that ions other than Cl^- participate in cholinergic excitation of these cells. The Cl^- efflux caused by ACh indicates that Cl^- is not distributed passively and that its equilibrium potential (E_{Cl}) is less negative than the E_m. If we assume that E_{ACh} is equal to E_{Cl}, then $[Cl]_i = [Cl]_o \exp(E_{Cl}\ F/RT)$ where $[Cl]_i$ is the internal concentration of Cl^-; $[Cl^-]_o$, its external concentration; E_{Cl}, the equilibrium potential of chloride; *F*, the faraday; *R*, the gas constant; and *T*, the absolute temperature. Since E_{ACh} is about −25 mv, $[Cl]_i$ would be about 43 mmole/liter, that is, two times greater than the expected for a passive distribution. To explain this high $[Cl]_i$ we have to postulate the existence of an inward pump of Cl^-, as described for the squid giant axon (*9*). The $[Cl]_i$ calculated from E_{ACh} in *D*-neurons is higher than that of 27.5 mmole/liter determined by Kerkut and Meech (see *10*) in *D*-cells of *Helix aspersa*.

The probable existence in the snail nervous system of two kinds of chloride pumps must be emphasized. We have presented evidence that *H*-neurons extrude Cl^- actively (*5*). In both *H*- and *D*-cells ACh increases the membrane permeability to Cl^-; however, the effect of this synaptic transmitter on each of them is different, due to the existing electrochemical gradients: in *D*-neurons Cl^- tends to leave the cell under the action of ACh, thus depolarizing the neuron; while in *H*-neurons Cl^- moves into the neuron and hyperpolarizes it. Thus, the effect of a synaptic transmitter depends not only on the permeability change which it elicits but also on the intracellular ionic content. It should be remembered that *D*- and *H*-neurons differ not only in their $[Cl]_i$ but in their ACh-receptors as well; only *D*-neuron receptors are blocked by hexamethonium (*11*).

Table 2. Values of E_{ACh} (millivolts) in *CILDA* neurons bathed with different solutions. The values in parentheses are the means ± the standard errors. The mean change (calculated for paired data) between the E_{ACh}'s in the control and low Na^+, chloride-free solutions was 18.3 ± 2.53 mv ($P < .001$); between those in the control and chloride-free solutions, 1.75 ± 0.93 mv ($P > .05$); between those in the control and 24 mM Ca^+ solutions, 0.40 ± .98 mv ($P > .1$); and between those in the control and 15 m*M* K^+ solutions, 0 mv.

Solution				
Control	Low Na^+, Cl-free	Cl-free	24 m*M* Ca^+	15 m*M* K^+
−10.0	−25.0	−15.0	−14.0	−10.0
0	−21.0	0	0	0
− 2.0	− 8.0	− 2.0		− 2.0
0	−20.0	0		
− 5.0	−20.0	− 2.0	− 5.0	
0	−30.0			
0	−16.0	− 4.0		
3.0	−20.0	0		
− 2.0			0	
0			0	0
12.0		12.0		
(−0.40±1.40)				
	(−20.0±2.30)			
		(−1.38±2.61)		
			(−3.80±2.7)	
				(−3.00±2.40)

If in *CILDA* neurons Na^+ is the only ion involved in the origin of the cholinergic excitation, the E_{ACh} should be more positive than 0 mv, the value reported here. Two possible explanations are first, that there was an error in the determination of E_{ACh}. The E_{ACh} was obtained by extrapolation from values of E_m between −80 and −30 mv with a linear relationship assumed to hold between the amplitude of the ACh-potential and E_m over the entire range. Nonlinearity could introduce a serious error. Second, participation of K^+ in the ACh-potential would make E_{ACh} less positive than the equilibrium potential of Na^+. Potassium ions may participate when the cell is depolarized to −35 mv or more. At this E_m snail neurons show delayed rectification (*12*), suggesting that the permeability to K^+ is increased (*13*). The lack of change in E_{ACh} when the preparation was bathed with a saline containing 15 mmole of K^+ per liter could be explained by a limited participation of

K^+. In any case, with our results it is not possible to rule out a small participation of K^+.

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14. Supported by grants of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina, and the United States Air Force Office of Scientific Research (grant AF-AFOSR 656-65).
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27 February 1967

Sulfite Oxidase Deficiency in Man: Demonstration of the Enzymatic Defect

Abstract. *An infant who died with neurological abnormalities, mental retardation, and dislocated ocular lenses excreted in his urine abnormally large amounts of* S-*sulfo*-L-*cysteine, sulfite, and thiosulfate and virtually no inorganic sulfate. The present report establishes the occurrence of an enzymatic defect in this infant. His liver, brain, and kidney specifically lacked sulfite oxidase activity. Deficiency of sulfite oxidase, which has not apparently been described in man, provides a reasonable explanation for the abnormalities in this infant.*

We have described (*1*) a male infant with a disorder of metabolism which we believe was previously unrecognized. At birth he had neurological abnormalities which progressed until he was virtually decerebrate. Dislocated ocular lenses were noted at age 1 year. He died at 32 months. Three of seven siblings were born with neurological abnormalities and died in early infancy for undefined reasons. The remaining siblings and the parents appear normal on clinical examination and have not yet been shown to have any disorders of their metabolism. The patient's urine contained greatly elevated concentrations of *S*-sulfo-L-cysteine, sulfite, and thiosulfate and virtually no inorganic sulfate (*2*). The abnormal chemical findings are readily reconciled by postulation of a deficiency of activity of sulfite oxidase, the enzyme that catalyzes the conversion of sulfite to inorganic sulfate. In the presence of such a deficiency, sulfate excretion would be abnormally low, and sulfite would accumulate. The raised concentration of sulfite in body fluids could lead secondarily, by way of known reactions, to the formation of abnormal amounts of *S*-sulfo-L-cysteine (*3*) and thiosulfate (*4*). We now report direct evidence that the patient was indeed markedly deficient in sulfite oxidase activity.

Sulfite oxidase [sulfite : oxygen oxidoreductase E.C. 1.8.3.1 (*5*)] of mammals has been studied by Fridovich and Handler and their associates (*6*, *7*). Their methods, with minor modifications, were used for our study of tissues obtained from the patient and controls at postmortem examinations and stored at −50°C. To prepare an acetone powder, a tissue was minced in at least 15 times its weight of acetone at −50°C and thoroughly homogenized in a Waring Blendor. The suspension was filtered, and the residue was washed successively with acetone and dry, peroxide-free ethyl ether at −50°C. The resulting powder was dried at reduced pressure and stored at 2°C. For extraction, the powder was stirred at 0°C for 2 hours with 0.05*M* potassium phosphate buffer, *p*H 7.8, containing 0.005 percent Versene Fe-3 (*8*), 1 ml of buffer being used for each 100 mg of powder. Sulfite oxidase activity was assayed by determination of the sulfite- and enzyme-dependent reduction of cytochrome *c*. Absorbance changes at 550.5 mμ (A_{550}) were measured with a Beckman model DU spectrophotometer. The standard assay mixture, volume 3.1 ml, included: tris-HCl buffer, *p*H 7.6, 100 μmole; cytochrome *c* (horse heart, Type VI, Sigma Chemical Co.), 2.6 mg; Na_2SO_3, 10 μmole; Versene Fe-3, 5μg, and enzyme (20 to 50 units). The solution was contained in a cuvette with a 1.0-cm light path and maintained at room temperature (19° to 20°C). The reaction was started by addition of enzyme. The reference solution against which absorbance of the assay mixture was determined contained only tris-HCl buffer and cytochrome *c*. Reaction rate was determined during the period when extent of reaction varied linearly with time, generally between 2 and 12 minutes after enzyme addition. The observed reaction rate was corrected by subtraction of two blank values. One was determined by incubation of enzyme and cytochrome *c* in the absence of added sulfite; the other, determined by omission of enzyme, represented the slow nonenzymatic reduction of cytochrome *c* by sulfite (0.001 absorbance unit per minute). A unit of enzyme activity was considered that amount of enzyme required to catalyze a change of 0.001 absorbance unit per minute under the standard conditions. Protein was measured by the method of Lowry as described by Layne (*9*). All specific activities are expressed as activity per milligram of protein.

Sulfite oxidase activity was readily demonstrable in crude extracts made from acetone powders of human liver. With some extracts a slow rate of enzyme-dependent reduction of cytochrome *c* was observed in the absence of added sulfite. To minimize or prevent this reduction, extracts were routinely purified by passage over a column of Sephadex G-25, maintained at 2°C, equilibrated with tris-HCl buffer, 0.05*M*, *p*H 7.6, containing 0.005 percent Versene Fe-3. Sulfite oxidase was quantitatively recovered after elution with the same buffer without significant change in specific activity.

In tris buffer, the sulfite oxidase activity changed little from *p*H 7.6 to 9.1. The reaction rate at *p*H 6.9 was 60 percent of that at *p*H 7.6. The