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BIOCHEMICAL AND PHARMACOLOGICAL ASPECTS OF THE SYNAPSES OF THE SQUID STELLATE GANGLION*

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Abstract—The giant axon and isolated single giant synapses from the squid stellate ganglion were analyzed for acetylcholine (ACh), choline acetylase, and acetylcholinesterase (ACh-esterase). ACh, choline acetylase, and ACh-esterase are all present in significant quantities in the giant axon of the squid. Isolated single giant (distal) synapses from the squid stellate ganglion were found to have much higher concentrations of ACh, choline acetylase, and ACh-esterase than the giant axon. ACh and related compounds failed to affect the synapse when it was prepared in the usual way. Effects were seen, however, in carefully cleaned preparations from which surrounding connective tissue was removed; 5×10^{-2} M ACh plus 10^{-4} M physostigmine produced a reversible block of synaptic transmission in two of eight experiments, and an irreversible block was produced in the other six; 5×10^{-2} M choline plus 10^{-4} M physostigmine had no effect on the electrical activity of the synapse. The significance of the presence of the ACh system in the giant synapse and its structural and functional interrelationships are discussed.

MANY investigators have studied the synapse between the second- and the third-order giant axons of the squid, referred to as the distal or giant synapse of the stellate ganglion.[1–3] Its enormous size greatly facilitates the study of certain aspects of the synaptic mechanism, especially those requiring methods of electrophysiology. While these aspects have been thoroughly investigated, not much is known about the biochemical aspects of the preparation.[4] None of the substances referred to as physiological transmitters has been found to have any notable pharmacological effect on transmission in this synapse. In particular, compounds acting on the acetylcholine (ACh) system have failed to affect the pre- and postsynaptic events. This is surprising in view of the evidence accumulated that ACh plays an essential role in the permeability cycle in excitable membranes and in view of the presence of ACh-esterase in this ganglion, demonstrated a long time ago.[5] Since there is a large amount of connective tissue surrounding the synapse, it is likely that drugs externally applied do not reach the active membrane. This structural barrier may account for the lack of action of the drugs tested.

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For studying the role of ACh in either conduction or transmission, it is desirable first to obtain information on the presence, concentration, and distribution of ACh, of choline acetylase, of the ACh-receptor, and of cholinesterase. A second step would require the study of the interrelation of these components with the synaptic function by means of the more elaborate and refined techniques available today. In addition to the observations reported here, some biochemical and histochemical investigations of the distribution and localization of cholinesterase, ACh, and choline acetylase are reported elsewhere.[4, 6]

METHODS

The stellate ganglia of the squid (*Loligo pealii*) together with 2 or 3 cm of the pallial nerve and 3 or 4 cm of the last stellar nerve, where dissected by approximately the same technique described previously.[7] The fin nerve was always completely removed. Immediately after dissection, the preparations were placed in continuously oxygenated filtered seawater. The preparations were mounted in a chamber kindly loaned to us by Dr. S. Bryant. The stellate ganglion was placed in a cylindrical chamber about 11 mm in diameter, 4 mm deep, and open at the top. Outlets on opposite sides of this chamber for the pre- and postsynaptic nerves were sealed from the central chamber with petroleum jelly. The threads tied to the ends of the nerves were fastened with beeswax so that there was a mild tension on the preparation; this did not affect the electrical properties. Both pre- and postsynaptic nerves rested on a pair of silver-wire stimulating electrodes. The nerve chambers were sealed off with parafilm and petroleum jelly to prevent the nerves from drying. The chamber containing the ganglion was continuously perfused with oxygenated filtered seawater with or without the addition of a drug. When high drug concentrations were used, the proper amount of distilled water was added to the seawater to maintain normal osmolarity. The volume between the three-way stopcock and the ganglion chamber was so small that the change of a solution was effected in 2 or 3 sec.

Two types of techniques were used in an attempt to remove some of the permeability barriers surrounding the giant synapse to determine whether effects of cholinergic drugs could then be demonstrated. One technique was the physical removal, by careful dissection, of the connective and nervous tissue surrounding the giant synapse. This tissue forms a part of the stellate ganglion which is normally left intact when the preparation is removed from the squid. The technique is described further in the next paragraph. The other technique was the attempt to reduce the barriers by chemical treatment with surface-active agents (cetyltrimethylammonium chloride or dioctyl sodium sulfosuccinate) or with cottonmouth moccasin venom. It has already been demonstrated that pretreatment of nerves with these two types of agents can permit curare, ACh, and other cholinergic drugs to produce reversible effects on conduction in preparations which, without pretreatment, do not react to these drugs at all even in extremely high concentrations.[8–12] The compounds used were curare, tetracaine, dibucaine, hexamethonium, benzoquinonium, physostigmine, acetylcholine, and choline. The last two were used in combination with or without physostigmine. When pretreatment was used, the synapse was tested for electrical activity, exposed for 30 min to the pretreatment agent, washed in seawater for 30 min, tested for electrical activity, and then exposed to the compound under investigation.

In experiments where the surrounding tissue was removed for permitting easier access of compounds applied externally to the synaptic junction, the removal was done after the preparation was mounted in the chamber. The preparation was always mounted with the side facing up on which the fin nerve had formerly rested. It might be noted here that in Bryant's study of the effect of drugs on the squid giant synapse the fin nerve was not removed in many of the experiments.[3] In the carefully dissected preparations of the present study, as much as possible of the tissue overlying the giant synapse was removed. About one third of these preparations had to be discarded because they were damaged by the removal process.

Glass microelectrodes filled with 3 M KCl, with resistances between 7 and 15 megohms, were used to impale the axons. The postsynaptic axon was impaled at a point just before it bends towards the proximal synapse, so that the electrode was either in or very close to the distal (giant) synaptic region. The presynaptic axon was usually impaled about 2 mm away from the giant synapse, because the diameter at that point is usually greater than at the synapse. Even so, it was difficult to impale and to maintain an impalement through a change in the perfusing solution; therefore, in quite a few experiments it was impossible to obtain measurements of the presynaptic spike, as will be shown later. Because of its larger size, the postsynaptic axon could be easily impaled and an impalement maintained through solution changes.

For testing the preparation, the presynaptic axon was stimulated supramaximally with 1/10 msec-duration pulses at a frequency of 100 stimuli/sec until the postsynaptic spike was eliminated. The resulting fatigue picture on a storage oscilloscope was photographed. The amplitude of the excitatory postsynaptic potential (EPSP) was defined as the amplitude of the first EPSP after the last spike. This definition was chosen because it is impossible to measure the amplitude of the EPSP while spikes are still being initiated, since the spikes begin while the EPSP is still rising. Therefore, it is impossible to discern a small decrease in the initial EPSP except perhaps in terms of a decreased fatigue time. Larger decreases in the amplitude of the EPSP, which prevent postsynaptic spike initiation with orthodromic stimulation, are easily measured. The amplitude of the spike was taken as the amplitude of the first spike.

For the enzyme and ACh studies, the stellate ganglion was separated into the proximal and distal synapse, and each of them was tested for ACh-esterase, cholinesterase, ACh, and choline acetylase. The Hestrin technique[13] was used for the determination of esterase activity. ACh and butyrylcholine were used as substrates. The pharmacological identification of ACh depended on the test originally proposed by Chang and Gaddum,[14] with the frog rectus abdominis.[15, 16] Choline acetylasea ctivity was determined according to the method described by Berman *et al.*[17] with a modified test mixture.[18] The composition of the incubation mixture (final volume 0·5 ml) was as follows: CoA, 0·5 μmole/ml, 0·2 ml; dipotassium acetylphosphate, 0·08 ml of a 0·5 M solution; phosphotransacetylase, $\sim$ 30 units/ml, 0·05 ml; choline chloride, 500 μmoles/ml, 0·04 ml; tetraethyl pyrophosphate, 50 μmoles/ml, 0·02 ml; magnesium chloride, 250 μmoles/ml, 0·02 ml; L-cysteine hydrochloride (neutralized) 950 μmoles/ml, 0·04 ml; potassium phosphate buffer (pH 7), 500 μmoles/ml, 0·02 ml; potassium chloride, 1330 μmoles/ml, 0·03 ml.

RESULTS

As may be seen in Table 1, there is a significant increase in ACh-esterase activity

in the distal synaptic region as compared to that of the axon. The proximal synapse, which is the origin of the third-order neurons and axons, shows less esteratic activity than that of the distal synapse but has significantly higher activity than the giant axon. When butyrylcholine instead of ACh is used as a substrate in order to determine the specificity of the enzyme, the lower rate of its hydrolysis indicates that most of the enzyme present is acetylcholinesterase.

TABLE 1. CONCENTRATION AND DISTRIBUTION OF ACh, CHOLINE ACETYLASE, AND CHOLINESTERASE IN THE STELLATE GANGLION OF SQUID

Tissue	ACh (μmoles/g)	Choline acetylase (μmoles ACh formed/g/hr)	Cholinesterase (μmoles ACh split/g/hr)	Cholinesterase (μmoles butyryl-choline split/g/hr)
Giant axon	0·020 ± 0·003 (7)	1·8 ± 0·23 (4)	2·1 ± 0·0 (15)	
Stellate ganglion	0·077 ± 0·020 (4)	36·0 ± 1·6 (4)		
Proximal synapse	0·018 ± 0·001 (3)	9·9 ± 3·56 (3)	47·2 ± 10·5 (4)	4·4 ± 0·8 (4)
Distal synapse	0·18 ± 0·04 (3)	59·9 ± 4·0 (6)	806·0 ± 72·7 (4)	440·0 ± 21·8 (4)

All results are presented as means ± standard errors.* Number in brackets indicates number of experiments. Test mixture used for determining choline acetylase is described under Methods.

* Standard error = $\sqrt{[\Sigma d^2/n(n-1)]}$.

Table 1 also shows the distribution of ACh and choline acetylase throughout the synaptic region. The distal synaptic region shows the highest accumulation of ACh as compared to that of the giant axon itself or the proximal synapse. The ACh content of the proximal synapse is almost identical with that of the axon itself. While the distal synapse appears to have a high concentration of choline acetylase, the proximal synapse shows much less activity. In spite of its almost identical ACh concentration, it shows a much higher choline acetylase activity than the giant axon. It thus appears that there is an accumulation of ACh, choline acetylase, and cholinesterase in the synaptic region, one of the requirements for attributing a role to ACh in the membranes of this preparation. This is in contrast to the neuropharmacological evidence so far, which shows that compounds known to act on the cholinergic transmitter mechanism show only little or no effect at all.[3] One major difficulty in this kind of investigation is that the large amounts of connective tissue and other nonsynaptic components surrounding the synapse offer a formidable permeability barrier to compounds applied from the outside. The sensitivity of the synapse to manipulation makes it difficult, although not impossible, to dissect a preparation free from non-essential tissue, in order to permit an easier access of compounds to the active sites.

Pretreatment of the preparation with surface-active agents or with cottonmouth moccasin venom failed to sensitize the synapse to drug action. Fifteen experiments were done with cetyltrimethylammonium chloride, dioctyl sodium sulfosuccinate (Aerosol OT), or cottonmouth moccasin venom pretreatment, followed by treatment with curare or ACh plus eserine. In three of these experiments no changes were seen. In the other twelve experiments either a reduction in amplitude or a complete block

of the postsynaptic (giant) axonal action potential was seen even with antidromic stimulation. This confirmed earlier work demonstrating that ACh or curare can block axonal conduction in squid giant axons pretreatment with a surface-active agent or with venom.[9, 10] However, no effect of ACh or curare directly on the synapse could be seen in the pretreatment preparations. Careful dissection of the synaptic region seems to be a more promising technique than pretreatment for sensitizing the synapse to drug action, because of the large amount of connective and nervous tissue still surrounding the synapse after it has been removed from the squid in the usual manner. Tables 2 and 3 show the effects of several drugs on the carefully dissected preparation.

TABLE 2. THE EFFECTS OF INHIBITORS OF THE ACh SYSTEM ON ELECTRICAL ACTIVITY OF THE CAREFULLY DISSECTED GIANT SYNAPSE

Drug	Conc. (M)	Time (min)	% Decrease in spike magnitude: Postsynaptic spike		EPSP		Presynaptic spike	
Curare	5×10^{-3}	75	0					
Curare	5×10^{-3}	60	100	I				
Curare	5×10^{-2}	71	70	R				
Tetracaine	5×10^{-4}	15	25	R				
Tetracaine	5×10^{-4}	25	100	I*	100	R	40	R
Tetracaine	5×10^{-4}	30	100	I*	100	I		
Tetracaine	5×10^{-4}	30	100	R*	80	R		
Tetracaine	5×10^{-4}	60	20	R	0			
Tetracaine	5×10^{-4}	82	15	R	0			
Dibucaine	5×10^{-5}	65	20	I	45	I		
Dibucaine	5×10^{-5}	65	20	R	20	R		
Dibucaine	5×10^{-4}	16	100	I*	100	I		
Dibucaine	$7{\cdot}5 \times 10^{-5}$	56	100	R*	0			
Eserine	$7{\cdot}5 \times 10^{-2}$	50	15	R	75	I		
Eserine	$7{\cdot}5 \times 10^{-2}$	16	20	I	65	R		
Eserine	$7{\cdot}5 \times 10^{-2}$	16	25	I	10	R		
Hexamethonium	4×10^{-2}	60	20	I	0		20	
Hexamethonium	6×10^{-2}	65	60	R	65	R		

Figures express the per cent decrease in magnitude compared to the control in individual experiments on individual preparations. R means that the decrease was at least 50 per cent reversible when the preparation was returned to seawater. I means the decrease was irreversible. Where neither I nor R appears, the reversibility was not checked. Where no EPSP values are given, only external electrodes were used. Where no presynaptic spike values are given, the presynaptic axon was not impaled with a microelectrode. In most experiments where the postsynaptic spike was blocked with presynaptic stimulation, the postsynaptic axon was stimulated directly, and the spikes thus obtained are given in this table. However, in those cases marked with an asterisk*, direct stimulation was not tried, so the postsynaptic axon may not have been blocked.

High concentrations of ACh did seem to produce an effect on the synaptic transmission in preparations in which connective tissue was removed (Table 3). What appears to be a reversible block of the synapse has been seen in two of eight experiments where 5×10^{-2} M ACh with 10^{-4} M eserine has been tried. Irreversible block occurred in the other six experiments. One of the two reversible experiments is illustrated in Fig. 1. The EPSP progressively decreases in the presence of ACh; eventually it disappears. It returns during recovery in normal seawater. It must be noted that the

presynaptic spike is also reduced. This effect of ACh on the presynaptic axon might possibly account for the reduction and block of the EPSP, since other investigators have shown that a reduction in the presynaptic spike causes a reduction in the EPSP.[1, 2, 19] It would be expected that ACh should affect the events in both membranes, if it has the postulated role. It should also be noted in Table 3 that ACh reduces the

TABLE 3. THE EFFECT OF ACh ON THE ELECTRICAL ACTIVITY OF THE CAREFULLY DISSECTED GIANT SYNAPSE[a]

Drug	Conc. (M)	Time (min)	% Decrease in spike magnitude					
			Postsynaptic spike		EPSP		Presynaptic spike	
ACh	5×10^{-2}	16	20	I	100	R		
	5×10^{-2}	90	25	I				
ACh + eserine (10^{-4} M)	5×10^{-2}	14	50	R	100	I		
	5×10^{-2}	16	100	R*	60	R		
	5×10^{-2}	18	20	R	100	I	100	I
	5×10^{-2}	18	100	R*	100	R	20	R
	5×10^{-2}	20	20	R	100	I		
	5×10^{-2}	20	40	R	100	I		
	5×10^{-2}	25	100	I	100	I		
	5×10^{-2}	25	40	I	100	I		
Choline + eserine (10^{-4} M)	5×10^{-2}	65	0		0			

[a] See footnote to Table 2.

postsynaptic spike, often reversibly. In any event, even though the exact site of action is uncertain, for the first time an effect of ACh has been demonstrated on the giant synapse preparation although only in high concentrations and after partial removal of the tissue barriers. It appears that the giant synapse may be a unique preparation in that the permeability barriers surrounding it may be even greater than those surrounding the pre- and postsynaptic axons. Table 3 shows that in a control experiment, 5×10^{-2} M choline with 10^{-4} M eserine produces no change in either the EPSP or the spikes. Thus, the effects observed must be attributed to a specific action of ACh.

DISCUSSION

The data presented show that ACh, choline acetylase, and ACh-esterase are present in the axons and the synapses of the stellate ganglion of the squid. The concentrations of the two enzymes as well as of the ester itself are very much higher at the level of the synapse than in the pre- and postsynaptic axons.[4] Although it has been repeatedly stressed that the presence of the ACh system is not sufficient evidence for the role postulated, the knowledge accumulated in favor of the primary role of ACh in the permeability cycle of excitable membranes makes it difficult to deny the same role in the membranes of the squid ganglia, especially in view of the evidence that the system is present there.

Such a role has been questioned, however, in view of the failure of compounds, known to act on the ACh system, to affect this synapse.[4] This failure obviously

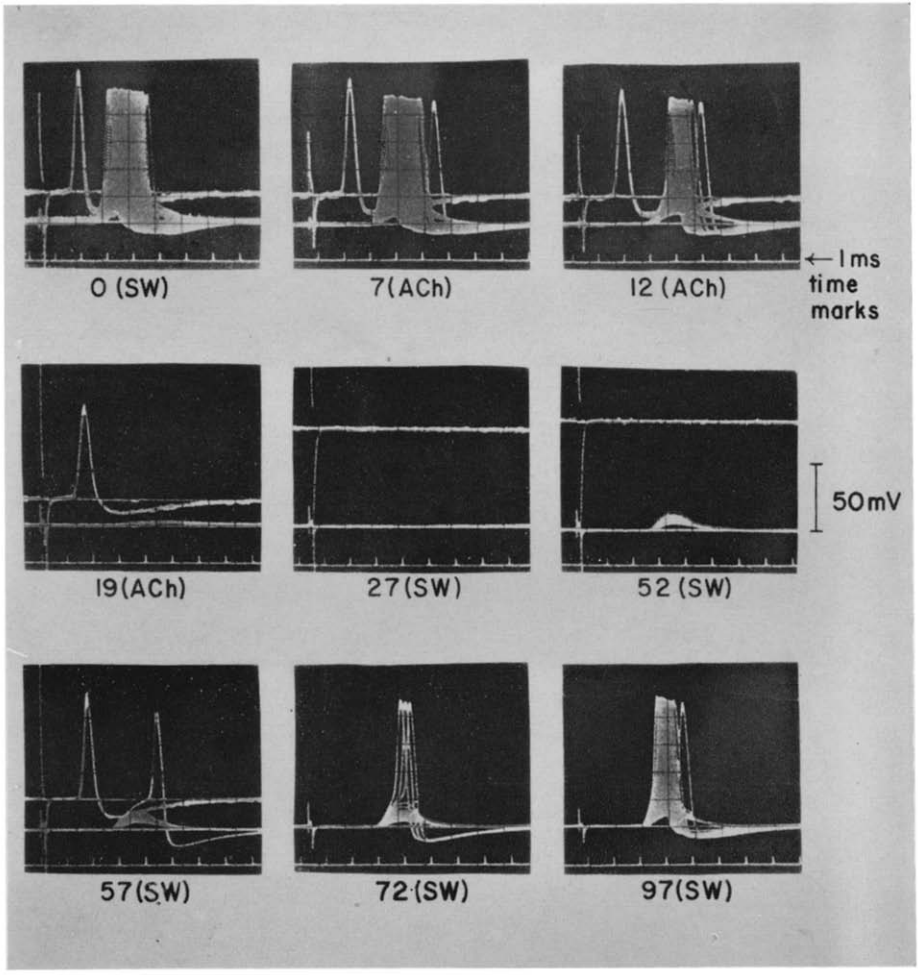

FIG. 1. The effect of 5×10^{-2} M ACh plus 10^{-4} M eserine on the giant synapse. The upper tracing is the recording from the presynaptic axon; in the middle is the recording from the postsynaptic axon. The bottom tracing shows 1 msec time marks. Time in minutes is given under each photograph. Perfusion was changed from seawater (SW) to seawater containing ACh and eserine at time = 2 min and back to plain seawater at time = 20 min. Stimuli were delivered to the presynaptic axon at 100/sec until fatigue occurred. Impalement of the presynaptic axon was lost at times 27, 52, 72, and 97 min.

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requires an explanation. A possible one is that, in contrast to many synaptic junctions, this particular one seems to be surrounded by large amounts of connective tissue which might conceivably form a structural barrier for the penetration of the compounds applied externally.

In the case of axons, in particular of the squid giant axon, the frog sciatic nerve, and the lobster nerve, two types of chemical pretreatment have been successfully tried to reduce these barriers: surface-active agents and snake venoms.[8–12] Neither of these treatments has been successful so far in the case of the giant synpase. However, removal of much of the connective tissue by dissection seems to be a more promising approach. Although, as would be expected, several preparations were damaged and became unusable, in several other cases specific effects were obtained. However, the percentage of successful experiments was still small and the concentrations required for obtaining effects were high. This latter factor is, of course, also true in the case of axons. Obviously, the barriers are reduced but not removed, and the situation is not comparable to that prevailing with the membranes at many junctions where no structural barrier exists. But the fact that several compounds including ACh in the presence of eserine did produce the postulated effects on the giant synapse suggests the value of refinement of this technique for demonstrating the role of ACh in the excitable membranes of this synapse.

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