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The effects of nitroprusside and putative agonists on guanylate cyclase activity in squid giant axons

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cGMP content of axoplasm from the giant axon of *Loligo forbesi* was investigated after subjecting the axon to various treatments. Repetitive electrical stimulation or depolarisation by high K^+ caused no change in cGMP content. Glutamate and serotonin were also without effect. The nicotinic agonist carbachol (100 μM) increased cGMP levels by 90% ($n = 5$). A large transient elevation of cGMP content was evoked by external nitroprusside (10 nM–20 μM) in intact axons. Nitroprusside injected into both extruded axoplasm and intact axons also increased cGMP content, the stimulation being considerably higher in intact axons where the axolemma was also present. Nitroprusside was also active in axons where the soluble cytoplasmic components were washed out by internal perfusion.

Introduction

The squid giant axon has proved an excellent preparation to investigate the electrical properties and membrane transport of neurons. The possible usefulness of the preparation in studying cyclic nucleotide metabolism has been recently revealed by studies on the hormonal control of cAMP metabolism [1,2] in both intact and perfused axons. No experiments are available so far on the cGMP metabolism in this preparation.

Our understanding of cGMP metabolism is far behind that of cAMP. Although two forms of guanylate cyclase, particulate and soluble [3–7] have been identified, it remains unclear for most tissues which enzyme is involved in the alteration of cGMP levels observed in intact cells, and also whether their cellular functions are regulated independently. Very little is known about the physiological factors which lead to a change in cGMP metabolism. There have been reports on the regulation of guanylate cyclase by hormones and neuromodulators [8–11]; but only the effects of ANF (Atrial Natriuretic Factor) on the particulate enzyme seems to be unequivocal [12–14].

However, there are certain physiological agents, such as L-arginine [15] and macromolecular activating factor [16], and non-physiological agents, such as nitro and nitroso compounds, which are able to activate soluble guanylate cyclase in cell-free preparations [17–20]. These compounds (nitroprusside, nitroglycerine, nitrosoguanydine, etc.) have proved useful in obtaining data on some properties of the enzyme.

In the present work cGMP metabolism has been investigated in the squid giant axon using

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Abbreviations: Mops, 4-morpholinepropanesulphonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)tetraacetic acid.

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nitroprusside as a tool to examine the cellular location and characteristics of guanylate cyclase. In this unique preparation it is possible to control the extracellular (intact axon) and intracellular (perfused axon) environment as well as to study a relatively intact axoplasm deprived of the axon membrane (extruded axoplasm). cGMP metabolism was also investigated in axons treated in various ways, aimed to mimick physiological conditions (electrical stimulation, possible neuromodulators, etc.).

Methods

Hindmost stellar giant axons of *Loligo forbesi* (diameter 500–1100 μm) were used in all experiments. Intact axon studies involved incubation in artificial sea water at room temperature (23° C), axoplasm was then extruded (see Ref. 21), weighed and homogenised in 300 μl ethanol for cGMP determination.

Axons for intracellular perfusion were first cleaned of adhering small nerve fibres and mounted in a horizontal chamber; this also permitted superfusion of the axon and measurement of membrane potential [22]. Internal perfusion was achieved by the coring method of Oikawa et al. [23]. The flow rate through the axon was maintained close to 5 μl/min by means of a Havard infusion pump. In general the first samples were taken 45–60 min after the onset of perfusion. The emerging perfusate was sampled every 10 min by collection directly into absolute ethanol for cGMP determination. Experiments were performed between 11° C and 13° C.

Homogenised axoplasm was centrifuged, the supernatant collected, the pellet resuspended in 2 parts ethanol/1 part H_2O and the process repeated. cGMP analysis was performed after drying down in an oven at 60° C and reconstitution in assay buffer. Recovery of cGMP from these methods was 80%, measured by injecting [^{3}H]cGMP into axoplasm and subjecting it to the normal extraction procedure. Ethanol samples from perfused axons were simply evaporated down and reconstituted into assay buffer (^{3}H recovery of 100%).

Standard external solution (mM): NaCl, 400; $MgCl_2$, 100; $CaCl_2$, 5; $NaHCO_3$, 2.5; pH 7.8. Where high K concentration was used Na^+ was replaced isosmotically by K^+.

Internal solution composition (mM); K_2HPO_4, 200; taurine, 300; Mops, 100; NaCl, 20; $MgCl_2$, 10; EGTA, 5; $CaCl_2$, 1.5; Phenol red, 0.5. pH adjusted to 6.8 with KOH. Osmolarity adjusted to 980 mosM with sucrose. In all experiments 8 μM carbonyl cyanide *m*-chlorophenylhydrazone (stock solution of 8 mM in dimethylsulfoxide) was included in the perfusate, and Na_2GTP was added directly.

Materials. cGMP radioimmunoassay kits from Amersham International. Serotonin (creatinine sulphate complex), ATP and GTP were from Sigma; all other chemicals were Analar grade or equivalent. Mean values of duplicate cGMP determinations from intact axons are given, whilst both duplicate determinations are given from perfused axons.

Results

cGMP levels in intact axons

cGMP levels of squid axoplasm determined after incubation for 30 min in standard external solution were in the range of 30–200 pmol/g (45 axons), with an average of 84 ($\pm$15 (S.D.)) pmol/g. Although cGMP content varied between animals a pair of axons from the same animal contained the same amount of cGMP, thus in all experiments one axon (or a part of an axon) from the same animal was used as a control when studying the effect of different conditions. Due to this variability, results are expressed relative to control.

Table I shows the cGMP content of axons after different treatments. Removing calcium from the bathing solution did not affect the cGMP content of axons. Neither repetitive depolarisation by electrical stimulation or prolonged depolarisation by 300 mM external K^+ nor increasing external Ca^{2+} concentration from 10 mM to 100 mM (by isomotic replacement of Mg^{2+}) had any clear influence on cGMP content. However, whilst these manipulations lead to an increase in free Ca^{2+}, we also found that in axons incubated in a medium where Li^+ replaced Na^+, a manipulation which also raises free Ca^{2+} [32,22], cGMP content fell to 50% of the control. The cGMP content of axons

TABLE I

EFFECT OF DIFFERENT CONDITIONS ON cGMP LEVEL IN INTACT AXON

Axons were incubated for 30 min at room temperature under various conditions, then axoplasm was extruded, weighed and homogenised in ethanol. cGMP content was determined by radioimmunoassay as described in methods. In high calcium media, magnesium was replaced by calcium isosmotically. The data shown are the means with S.E. *N*, number of determinations.

Condition	cGMP content relative to control	*N*
Control	1.0	
Ca^{2+}-free sea water	0.94 ± 0.11	4
Electrical stimulation (200 Hz)	0.85 ± 0.05	2
K^+ depolarization (300 mM K^+)	0.95 ± 0.08	3
Increasing external Ca^{2+} (100 mM)	0.88 ± 0.03	2
Li^+-sea water	0.54 ± 0.06	3

was increased 3-fold after phosphodiesterase was inhibited with 10 mM caffeine, rising from 81 ± 9.3 to 262 ± 15 pmol/g during a 30 min exposure ($n = 4$).

The effect of nitroprusside on intact axon

From the numerous nitroso-compounds known to activate guanylate cyclase we examined the effect of nitroprusside in order to reveal some of the characteristics of guanylate cyclase of squid axon. Due to the variation in cGMP content between axons from different squid we always examined the effects of nitroprusside by using axons dissected from the same squid. Two of these experiments are illustrated in Fig. 1. In the presence of nitroprusside there was a large increase in cGMP content. The effective concentrations of nitroprusside and the time-course of its effect in other experiments ($n = 3$) were also within the range shown in Fig. 1.

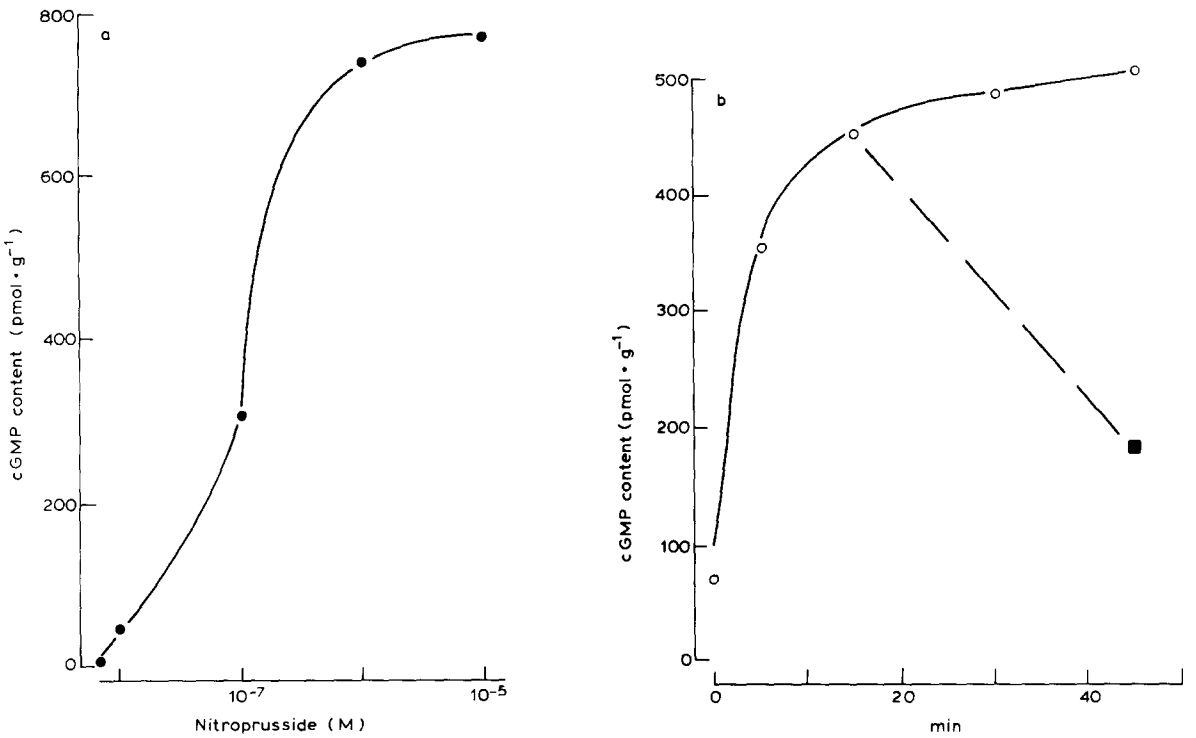

Fig. 1. (a) The increase of cGMP level in intact axon as a function of nitroprusside concentration. Axons from the same animal were divided and incubated for 30 min at room temperature in standard external medium containing different concentrations of nitroprusside. (b) Time-course of the effect of 10 nM nitroprusside on cGMP level of intact axon. Axons from the same squid were incubated with 10 nM nitroprusside for varying periods (open circles). After 15 min in the presence of nitroprusside one axon was washed a few times in media in the absence of nitroprusside for 30 min (closed square). At the end of each incubation axoplasm was extruded, homogenised in absolute ethanol and cGMP content determined as described in Methods.

The minimal concentration of nitroprusside found effective (10 nM) is well below that observed in most other preparations [24,25]. This effect seems to be reversible; if the axon is incubated for 15 min with nitroprusside and then subsequently washed several times with normal external medium, cGMP content fell by 60% after 30 min (Fig. 1B, closed square).

The effects of nitroprusside on perfused axons

In an attempt to examine cGMP production by plasmalemmal (particulate) guanylate cyclase, axons were cored of axoplasm and perfused intracellularly to wash out the cell interior [23]. Emerging perfusate samples (10 min collection period) were taken over 40 min after the onset of perfusion, by which time the axon interior (approx. volume 6 μl) had been continually washed with over 30-times its volume. Throughout the experiment 1 mM GTP was always present in the perfusate.

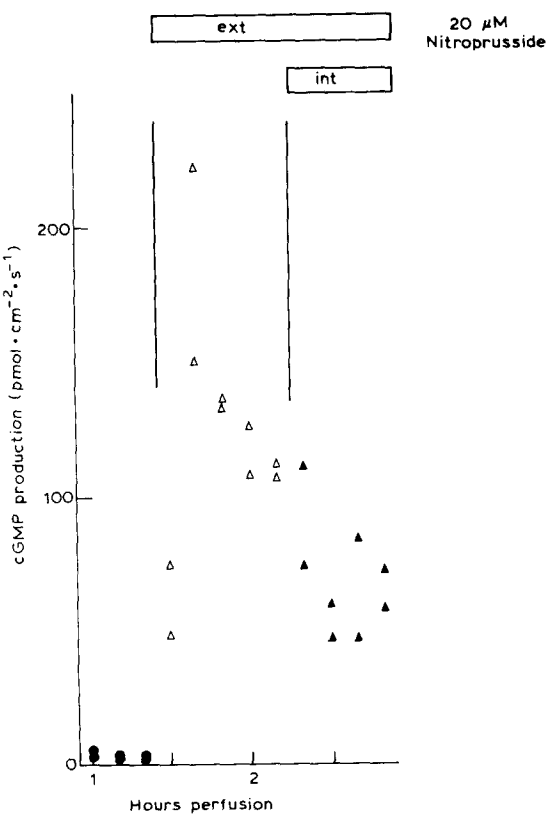

Fig. 2. The effects of external and internal nitroprusside on cGMP production in intracellularly perfused axons. Axon was perfused as detailed in Methods. Axon diameter; 1086 μm. External solution contained no caffeine. Perfusate contained 1 mM GTP and no ATP.

The effects of nitroprusside are illustrated in Fig. 2. Addition of nitroprusside to the external medium caused a large transient increase in the cGMP levels in the emerging perfusate. This rise in cGMP content would presumably reflect both the rates of guanylate cyclase production and phosphodiesterase breakdown. However, we found no phosphodiesterase activity under these conditions; this was determined by perfusing through a constant level of cGMP in the nominal absence of GTP. Under these conditions the perfusing levels of cGMP were the same as the emerging cGMP levels (data not shown). Therefore the elevation in cGMP content in response to nitroprusside can be regarded to be due to activation of guanylate cyclase, rather than a possible inhibition of phosphodiesterase activity. The maximal rate of cGMP production (185 $fmol \cdot cm^{-2} \cdot s^{-1}$) was achieved 20 min after the addition of external nitroprusside, thereafter the cGMP content in the perfusate decreased and was not significantly affected by the inclusion of nitroprusside in the perfusion medium, although still many times basal.

Injection of nitroprusside into intact axon and extruded axoplasm

Taking the unique advantage of the squid axon that its axoplasm can be simply extruded and a relatively intact cytoplasm without the axon membrane can be studied, we performed a few experiments in an attempt to localise the guanylate cyclase activated by nitroprusside.

Pairs of axons from the same squid (I, II, III) were divided into four and each subjected to different treatments as shown in Table II. One half-axon from each squid was incubated in standard external medium and served as a control. Another half-axon was incubated in standard external medium containing nitroprusside (100 nM). Nitroprusside, at a final concentration of 100 nM, was injected into an intact axon as described [21] and then incubated in normal medium. Finally, the axoplasm of the fourth half axon was extruded (extrusion removes close to 95% of axonal axoplasm [26]), sucked into the tip of a Pasteur pipette (to avoid drying out), then injected with nitroprus-

TABLE II

EFFECTS OF 100 nM NITROPRUSSIDE ON cGMP LEVELS WHEN INJECTED INTO INTACT AXON OR INTO EXTRUDED AXOPLASM

For each experiment two axons from the same squid were cut into two and each half-axon used for the different experimental conditions: Two half axons were incubated with or without (control) nitroprusside. Nitroprusside was injected into the third half-axon as described by Baker et al. [21]. Axoplasm from the fourth half-axon was extruded, sucked into a small pipette and injected with nitroprusside. Axons were incubated for 30 min at room temperature. cGMP contents are given in pmol/g, as well as relative to control (in brackets). n.d., not determined.

Squid	Control	Nitroprusside		
		external	internal	
			intact axon	extruded axoplasm
I	130 (1)	900 (6.9)	18500 (142)	450 (3.2)
II	90 (1)	3800 (42)	10326 (114)	900 (10.0)
III	110 (1)	n.d.	12000 (109)	800 (7.2)

TABLE III

EFFECTS OF CARBACHOL, SEROTONIN AND GLUTAMATE ON cGMP LEVELS IN INTACT AXONS

Pairs of intact axons were incubated in normal medium in the presence of 100 μM agonist. Axons from the same squid without agonist are controls. cGMP content is expressed in pmol/g as well as relative to control (in bracket). Mean value are given for serotonin and glutamate (each $n = 3$).

		Control	Test
Carbachol (100 μM)			
Squid	I	53	151 (2.8)
	II	50	145 (2.9)
			118 (2.36) [a]
	III	185	270 (1.45)
	IV	173	320 (1.8)
	V	93	60 (0.6)
+10 mM caffeine	VI	544	700 (1.2)
Serotonin (100 μM)		80	96 (1.2)
Glutamate (100 μM)		91	100 (1.1)

[a] In this experiment 50 μM carbachol was used.

side (final concentration; 100 nM) and left at room temperature. After 30 min the axoplasm of all four pieces were weighed and the cGMP content determined. The results (Table II) clearly show that nitroprusside when injected into an intact axon increased cGMP content more effectively than when added externally. Although the possibility remains that in the latter case the concentration of nitroprusside at the site of guanylate cyclase could be lower than in the case of a direct injection which might explain that difference. To exclude the possibility of an injection artifact we injected KCl into an axon (final concentration, 100 nM) and found no change in cGMP content.

A particularly interesting finding in these experiments is that the effect of nitroprusside is very mitigated when injected into extruded axoplasm. Injection into intact axon resulted in a more than 100-fold increase of cGMP content, this being at least 10-times more effective than the same amount of nitroprusside injected into extruded axoplasm. The increase in the latter case was even smaller than that caused by external nitroprusside in an intact piece of the same axon. The basal level of cGMP (without nitroprusside) in extruded axoplasm did not differ from that found in intact axon (data not shown).

The effect of some neurotransmitters and neuromodulators on cGMP content of squid axon

We have also performed a few preliminary experiments to see whether cGMP content in squid axons is influenced by substances known to act as neurotransmitters or neuromodulators in other systems (Table III). Glutamate, which is the putative neurotransmitter of the giant fibres in the squid [27], was found ineffective, so was serotonin which enhances cAMP content in the same preparation [1,2]. However, the nicotinic agonist carbachol (100 μM) was able to elevate cGMP level of axons in five out of six experiments (2.2-fold increase from controls in the absence of caffeine). In one experiment 10 mM caffeine was used to inhibit phosphodiesterase, under these conditions 100 μM carbachol was still able to further elevate the levels of cGMP. This effect might have physiological significance as acetylcholine synthesis as well as nicotinic receptors have been found in Schwann cells of the giant nerve fibre of the squid [28–30].

Discussion

cGMP content of the axoplasm of squid axon is in the range 30–200 pmol/g (mean ± S.D. (n = 45), 84 ± 15) as found for other tissues [9]. It was not changed when the axon was depolarized either by electrical (at 200 Hz) or chemical (300 mM K^+) depolarisations. These conditions lead to an increase of cGMP in barnacle muscle [31], an effect which does not seem to be generally characteristic for excitable tissues. There does not seem to be a direct effect of intracellular Ca^{2+} on cGMP levels, as depolarisation has been shown to bring about a steady increase in free Ca^{2+} [32,33,22]. When external Na^+ was replaced by Li^+, cGMP content was found to be half of controls (Table I). Under these conditions intracellular free Ca^{2+} is also raised [32,22]; however, from our results on depolarisation this is unlikely to be the reason for the change in cGMP. A possible direct effect of Li^+ on guanylate cyclase or on phosphodiesterase might deserve a more thorough study. The cGMP content of the axon results from the simultaneous function of guanylate cyclase and cGMP phosphodiesterase, when the latter was inhibited, by 10 mM caffeine, cGMP content was three times as much as in control axons (from 81 ± 9.3 to 262 ± 15 pmol/g, n = 4).

It has been shown in several studies that nitroso-containing compounds stimulated soluble guanylate cyclase, although the extent of stimulation varied in different preparations [17–20]. With partially purified enzyme the stimulatory effect of these compounds could be increased by the addition of hematin and hemoprotein and the formation of a nitroso-heme complex has been suggested as an obligate step in the activation of the enzyme [25,34]. In fact the heme moiety was present in soluble guanylate cyclase purified from bovine lung [35]. The variable responsiveness of soluble guanylate cyclase to nitroprusside found in different studies can perhaps be attributed to differences in the heme content of the enzyme purified from various tissues. We do not know whether heme is also a part of the soluble guanylase cyclase in squid axon, however, it would not give an explanation for the different extent of activation by injected nitroprusside between extruded axoplasm and intact axon (Table II). Extruded axoplasm retains its cytoskeleton intact, along with its cytoplasmic constituents [36], therefore it is very unlikely that possible cofactors of the soluble cytoplasmic enzymes are lost.

If we assume that the very considerable enzyme activation by injected nitroprusside into intact axon is mainly attributable to the soluble enzyme, then the decrease of the responsiveness found in extruded axoplasm can most likely be ascribed to the absence of the axon membrane. The possibility that the axolemma contains a factor (or factors) which can influence the function of the cytoplasmic guanylase cyclase cannot be excluded. It is also possible that a large proportion of the soluble guanylase cyclase is located in the close proximity of the axon membrane and retained within the 5–10 μm layer of cytoplasm still attached to the membrane after extrusion [26,36]. This possible compartimentation would again suggest an interaction of some kind between cytosolic guanylate cyclase and the axon membrane.

However, it seems perhaps more likely that in addition to the cytoplasmic enzyme squid axon contains a particulate enzyme located at or near the axolemma which is more sensitive to activation by nitroprusside. In previous experiments with purified enzymes it has remained unclear if the particulate enzyme could also respond to nitroprusside since most of the particulate preparations were conaminated with soluble enzyme [37,38], although a rather recent study on particulate enzyme showed no response to nitroso compounds [14]. The suggestion that in our experiments the particulate enzyme can also be activated by nitroprusside is supported by the experiments with perfused axon (Fig. 2); this shows that after 1.5 h internal perfusion nitroprusside was still able to enhance cGMP levels in the perfusate. Similar experiments on plasmalemmal serotonin-stimulated adenylate cyclase [2] clearly show that 20–30 min perfusion is sufficient to permit control of the soluble cytoplasmic constituents.

(If we use the data from Fig. 1a on intact axons incubated with 10 μM nitroprusside there is an increase in cGMP content of 253 $pmol \cdot g^{-1} \cdot min^{-1}$; for an axon diameter of 750 μm this would correspond to a guanylate cyclase rate of production of 79 $fmol \cdot cm^{-2} \cdot s^{-1}$. This is calcu-

lated on the assumption that phosphodiesterase activity is minimal and cyclase activity plasmalemmal in origin. However, we have a measure of phosphodiesterase activity from Fig. 1b which shows that after washout of 10 nM nitroprusside cGMP content fell from 450 pmol/g to 185 pmol/g over 30 min, presumably due to phosphodiesterase. This gives an estimate of 3 fmol $\cdot$ $cm^{-2} \cdot s^{-1}$. Thus, with 10 μM nitroprusside the rate of guanylate cyclase production in intact axons can be considered close to 82 fmol $\cdot$ $cm^{-2} \cdot s^{-1}$ ($79 + 3$ fmol $\cdot$ $cm^{-2} \cdot s^{-1}$), whilst perfused axons exposed to 20 μM nitroprusside (Fig. 2) can produce maximal rates of 185 fmol $\cdot$ $cm^{-2} \cdot s^{-1}$. Guanylate cyclase rates from perfused axons are therefore, surprisingly, close to twice those seen in intact axons which still retain their cytosolic constituents.)

The physiological role of the particulate enzyme is still unclear. Although there are reports suggesting a receptor-mediated activation of particulate guanylate cyclase most of the data are controversial (see Ref. 9). In intact squid axon glutamate, which is assumed to be the transmitter in the giant synapse [27], was without effect on cGMP content, just as was serotonin. The latter was suggested [39] to raise cGMP level of *Helix aspersa* neurones resulting in an increase of calcium current. This does not seem to be the case in squid axon where it caused no change in the level of cGMP, but elevated that of cAMP [1,2]. However, the cholinergic nicotinic agonist carbachol clearly enhanced cGMP content in intact axon. Although a more detailed study is needed to reveal the mechanism and the physiological significance of this effect, the finding seems to be consistent with the data of Villegas [27], which demonstrates that the squid giant nerve contains acetylcholine and this can be released from Schwann cells [28–30].

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