Biochemical Pharmacology, Vol. 20, pp. 2155–2162. Pergamon Press, 1971. Printed in Great Britain

DEPRESSANT EFFECT OF PUROMYCIN ON THE SYMPATHETIC GANGLIONIC FUNCTION

A STUDY OF THE POSSIBLE SITE AND MECHANISM OF ACTION*

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(*Received* 7 *December* 1970; *accepted* 2 *February* 1971)

Abstract—The possible site and mechanism of action of puromycin (PU) on sympathetic ganglion isolated *in vitro* are investigated. We have observed that: (1) the depressant effect of PU on functional activity is higher on active than on resting ganglia; (2) PU interference with the divalent cations of the medium is negligible; (3) PU depresses the post-ganglionic action potentials to a higher extent than those recorded from pre-ganglionic fibres; (4) synaptic potentials (recorded from curarised ganglia) are depressed to the same extent as post-ganglionic action potentials; (5) PU depresses the post-ganglionic response evoked by chemical stimulation with acetylcholine; (6) in the presence of PU, ganglia incubated with tritiated choline under sustained activity have a greater content of labelled acetylcholine. It is therefore suggested that PU exerts its action at both a pre- and a post-synaptic level.

IN A PREVIOUS investigation[1] we described the depressant effects, on the function of the isolated superior cervical ganglion of the rat, of puromycin (PU) and other inhibitors of protein synthesis and related compounds (cycloheximide, acetoxycycloheximide, puromycin-aminonucleoside). In view of our results, a correlation between the depression of the ganglionic function and the inhibition of protein synthesis would appear to be unlikely as well as the effect of abnormal polypeptides released by PU. The depressant effect was observed for drug concentrations similar to the intracerebral concentrations found to be effective on memory in experiments on mice by other workers.[2,3] Our results have clearly pointed to the fact that the impairment of memory, observed under such experimental conditions, might well result from a similar depression of the function of some neuronal system in the brain areas involved in memory processes. As a further development and integration of our work, we have carried out an analysis of the depressant effect exerted by PU with the purpose of elucidating its mechanism and site of action. We have explored in order, the effect of PU using the following experimental approaches: (1) active versus resting ganglia; (2) the levels of di-valent cations in the medium; (3) a comparison of the action potentials recorded simultaneously from pre- and post-ganglionic fibres; (4) the synaptic potentials of partially curarised ganglia; (5) the post-ganglionic response evoked by chemical stimulation with acetylcholine (ACh); (6) the synthesis and turnover of ACh in ganglia incubated with radioactive choline. The circumstantial evidence resulting from these observations indicates that PU exerts its action at both a pre- and a post-synaptic level.

* This research was financially supported by the Consiglio Nazionale delle Ricerche (Contract n.69.01669).

MATERIALS AND METHODS

Superior cervical ganglia of rat were excised, incubated and stimulated as described in our previous paper.[1] Depression of the electrical activity has been evaluated as a per cent difference in the amplitude from that recorded before application of PU. A 50 per cent reduction of the response was chosen as a suitable condition for evaluating the depressant effect.[1] *Resting ganglion:* the ganglion was kept at rest and was tested at 30-min intervals for 1 min at 1 stimulus/sec.

Pre- and post-ganglionic potentials were simultaneously recorded with one electrode on the pre-ganglionic and another on the post-ganglionic nerve trunk; the changes in the amplitude of the main pre-ganglionic component of the recording were followed. For this purpose the set-up usually employed for recording the post-ganglionic action potentials was modified by adding one more capillary tube containing a recording electrode and capped with a short piece of polyethylene tubing. The pre-ganglionic fibers were pulled through a tight bore across the polyethylene tubing before entering the glass capillary tube containing the stimulating electrode. The tip of the polyethylene tubing and the edges of the bore around the nerve trunk were sealed with vaseline.

Synaptic potentials (i.e. electrotonically propagated EPSP, according to Tauc[4]) were recorded by following the procedures described by Dunant and Dolivo[5] and Bures *et al.*;[6] 60 min before PU was tested, the ganglion was partially curarised (*d*-tubocurarine 225 μg/ml, final), then repetitive stimulation at 1/sec was carried out; recordings from the post-ganglionic trunk were made with a time constant of 1 sec (Tektronic Type 122 low-level preamplifier); the Hewlett–Packard 130 C oscilloscope was set as follows: DC amplifier and input; sweep-time 10 msec/cm.

The post-ganglionic response evoked by application of ACh was examined according to the following scheme, similar to that previously adopted by Nicolescu *et al.*[7] The ganglion was treated with prostigmine (5 μg/ml, final) and, 90 min later, was tested in the following way: first, a single repetitive stimulation (1 sec at 35/sec) was applied and the evoked asynchronous discharge recorded under the following conditions: preamplifier: time constant = 1 sec, voltage gain = 1000; oscilloscope: sweep time = 5 sec/cm and vertical sensitivity = 10mV/cm; second, PU (0·44–0·54 mM) was injected and 45 min later electrical stimulation was repeated and the discharge recorded. Then, after 10 min, ACh (1 μg/ml, final) was injected and the response was recorded: 15 min after washing, both electrical and ACh stimulation were repeated. A comparison was made, the response recorded after PU had been washed out being taken as a reference value (100 per cent). Medium containing prostigmine was used for washing.

Synthesis of labelled acetylcholine (ACh) and analytical procedure

After 30 min at rest the ganglion was stimulated at 5/sec for 30–45 min in order to check sustained post-ganglionic potentials. Then the incubation medium (1 ml) was replaced with the same medium (1 ml) which this time contained carrier Choline (Ch) (1·5 μg/ml)[8] plus radioactive Ch(10 μc/ml) (Choline chloride (methyl-T), specific activity 15·4 c/mM; Radiochem. Center Amersham) and supramaximal stimulation at 10/sec was started and continued for 2 hr. At the end of this incubation period, the mounted ganglion was transferred to ice-cold medium, the pre- and post-ganglionic fibres were cut away and the ganglion was rinsed quickly (twice) in 4 ml of ice-cold medium containing carrier Ch (20 μg/ml), then blotted on filter paper moistened with

saline, and dropped to the bottom of a conical tube (the homogenizer described by Larrabee *et al.*[9]) containing 200 μl of HCl solution, pH 4, plus 10 μg carrier Ch and 10 μg carrier ACh already immersed in a boiling water bath. After 10 min of this acid extraction at approximately 100°[10] the homogenizer was cooled in ice and the ganglion was homogenized by hand with a few round strokes of the conical fitting pestle. The homogenate was centrifuged for 15 min at 0–4° in the Servall centrifuge (rotor SS 34, 12,000 rev/min) and the supernatant transferred to a short conical tube. The pestle, homogenizer and sediment were rinsed twice with 200 μl HCl pH 4 and the washings, after centrifugation, were added to the supernatant: the final volume of the extract was about 600 μl. This extract was then dried overnight under vacuum in a desiccator containing both KOH and P_2O_5.The dry extract was redissolved with 30 μl of the buffer employed for electrophoresis and was analyzed as follows:

ACh was separated from Ch by electrophoresis (TL modification of Potter and Murphy procedure[11]) followed by chromatography[12] in the second direction. The analyses were carried out on a thin layer of cellulose powder prepared on glass plates, 20 × 20 cm (Merck), with an apparatus for TL electrophoresis (Camag, 4132 Muttenz —Switzerland). The electrophoresis buffer consisted of: 1·5 moles of acetic acid and 0·75 moles of formic acid per litre, pH 2; the chromatography solvent system was: 0·2 M ammonium acetate buffer pH 4·8 and acetone (1:4, v/v). The plate was finely sprayed with buffer, a small dry rectangular area being left at the start where 5 μl of a solution (electrophoresis buffer) containing 5 μg of carrier Ch and 5 μg of carrier ACh per μl were applied, followed by 10 μl of the extract. Electrophoresis was started immediately and run for about 30 min, at 1200 V and about 15 mA. The cooling fluid circulating in the apparatus was kept in the range of +2° to +6°. At the end of the run the plate was removed and dried in an air oven at 37°; then chromatography in the second direction was carried out at 20–22°, for about 3 hr, until the solvent had reached about 1–2 cm from the top of the layer. After drying, the spots were revealed by exposure to iodine vapours and outlined with a pencil. The plate was left in the open air until all the iodine had been removed (overnight). The outlined spots were collected by scraping the thin layer and transferring the powder into a counting vial. Each vial contained 250 μl of carrier Ch (2 mg/ml in methanol) plus 9 ml of Bray's scintillation fluid.[13] Samples were counted in a liquid scintillation spectrometer (Nuclear Chicago model 724) at +5°. For better precision each sample of radioactive spot was counted to a fixed count value (20,000 counts on channel C). Counting efficiency was evaluated by channel ratio and was 18–20 per cent.

Ethylenediamine-tetraacetic acid (EDTA) (Merck-Darmstad—Germany) was neutralised to pH 7 with NaOH.

Puromycin dihydrochloride (Nutritional Biochemicals Corporation, Cleveland, Ohio) was titrated to pH 6·5 with NaOH.

D-tubocurarine-chloride (Farmitalia, Milano).

Prostigmine (Roche, Milano).

Acetylcholine-chloride (Roche, Milano).

RESULTS

Resting versus active ganglion. The results of a typical experiment with PU (0·18 mM) applied to an active and a resting ganglion are illustrated in Fig. 1(a) and (b) and the effects measured in a series of experiments are reported in Table 1; the per

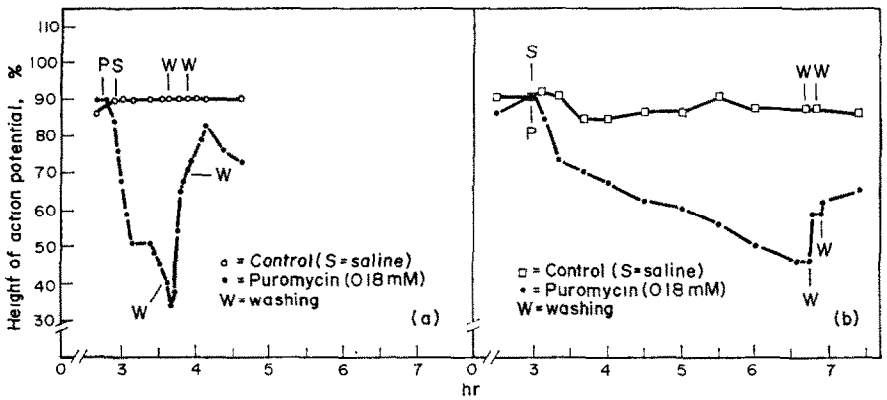

FIG. 1. Effect of PU on: (a) active ganglion (repetitive stimulation at 5/sec); (b) resting ganglion (tested at 30 min intervals by stimulating for 1 min at 1/sec). % Refers to the height of potentials at the time of PU addition.

cent depression induced by PU in the resting ganglion is lower, and is reached later, than that induced by the same concentrations of PU in the active ganglion.

Puromycin and divalent cations. The possible interaction of PU with the divalent cations of the medium has been taken into account. In fact, if the Ca level in the medium is lowered, or a complexing agent is added, a depression of synaptic transmission may result,[4,14] despite the fact that the firing threshold of the ganglionic cells has been lowered. As shown in Table 2, after PU had exerted its depressant effect, no recovery was observed when extra Ca was added to the medium (in order to compensate for a possible subtraction of Ca by PU). Conversely, the substitution of normal medium with low-Ca, or respectively low-Ca and -Mg, did not significantly affect the ganglionic response over 1 hr. In all these tests the amounts of divalent cations added to, or subtracted from the medium were chosen on the assumption that, in an extreme case, each PU molecule could bind four divalent cations. In a further series of experiments different amounts of a well known complexing agent, ethylenediamine-tetraacetic acid (EDTA) were added to the medium. As expected, only the high concentrations (which complexed a high percentage of the divalent cations)

TABLE 1. EFFECT OF PUROMYCIN ON ACTIVE AND ON RESTING GANGLION INCUBATED (*in vitro*)

	Final concentration (mM)	Time (min) for decrease to 50%* of height of post-ganglionic potentials	Height (%)* of post-ganglionic potentials 180 min after puromycin addition
Active† ganglion	0·09	—	62 ± 4 (4)
	0·18	54 ± 11 (6)	—
	0·54	4 ± 0·8 (4)	—
Resting‡ ganglion	0·18	—	71 ± 6 (5)
	0·54	24 ± 3 (3)	—

The reported values are the mean ± S.E.M.; numbers of experiments in parentheses.
* % is referred to the height of potentials at the time of puromycin addition.
† Repetitive supramaximal stimulation of preganglionic fibers (5/sec).
‡ Tested at 30 min intervals (stimulation for 1 min at 1/sec).

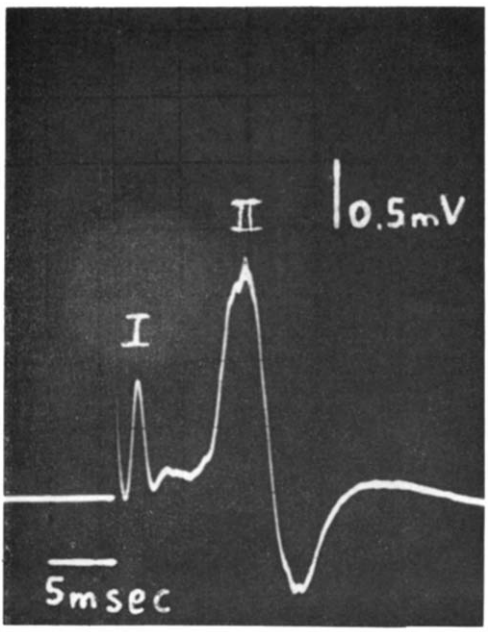

FIG. 2. Action potentials recorded from the pre-ganglionic (I) and post-ganglionic (II) nerve in response to supramaximal repetitive stimulation of the pre-ganglionic nerve (5/sec).

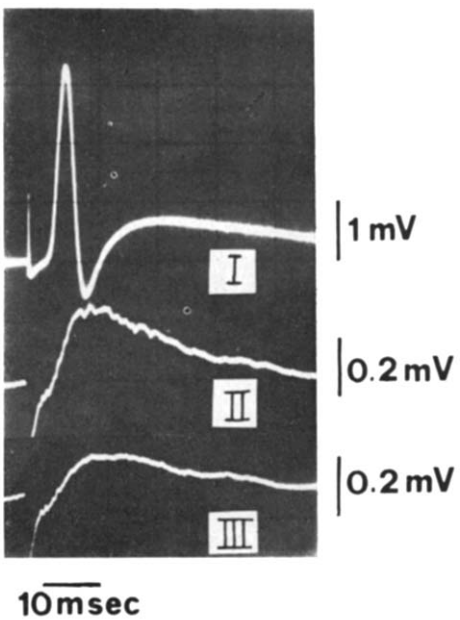

FIG. 3. Effect of PU on synaptic potential: I, action potential before curarisation; II, synaptic potential recorded 60 min after adding curare (225 μg/ml) and immediately before injection of PU 0·18 mM); III, synaptic potential recorded 40 min after adding 0·18 mM PU.

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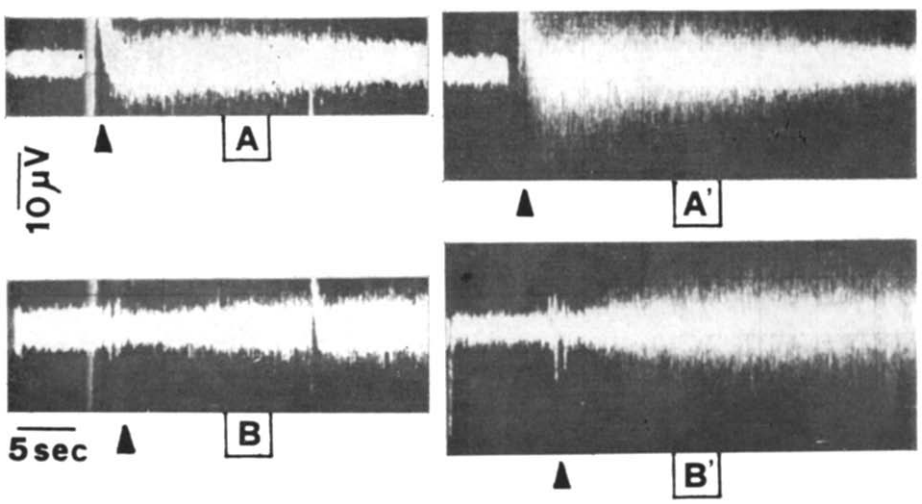

FIG. 4. Recording of the asynchronous discharge from post-ganglionic trunk of eserinised ganglion. In A and A′ the activity has been elicited by stimulation (35/sec for 1 sec); in B and B′ acetylcholine 2 μg/ml has been added to the medium (containing prostigmine 5 μg/ml). A and B show the activity 50 min after adding 0·54 mM PU; A′ and B′ show the recovered activity 13 min after washing out PU.

TABLE 2. PUROMYCIN AND DIVALENT CATIONS

	Final concentration of different components in the medium (mM)					
	Puromycin	Ca^{2+}	Mg^{2+}	EDTA (Na salt)	Height of post-ganglionic potentials (%)*	No. of experiments
Medium	—	2·22	1·20	—	No significant change over 1 hr	—
Medium + puromycin	0·18	2·22	1·20	—	50, after 54 min	6
Medium + puromycin + extra Ca†	0·18	3·02	1·20	—	No recovery over 1 hr	2
Low-Ca medium	—	1·40	1·20	—	No significant change over 1 hr	5
Low-Ca-Mg medium	—	1·68	0·93	—	No significant change over 1 hr	8
Medium + EDTA	—	2·22	1·20	1	No significant change over 1 hr	2
	—	2·22	1·20	2	90, after 30 min	1
	—	2·22	1·20	3	50, after 13 min	2

* % is referred to the height of post-ganglionic potentials before change to the indicated medium.
† Ca is added when 50 per cent inhibition is reached.

caused an evident depression of the post-ganglionic response. These effective EDTA concentrations lie between 2 and 3 mM, that is, they are 10–15 times the concentrations of PU which depress the ganglionic function.

Comparison of action potentials simultaneously recorded from both pre- and post-ganglionic trunks. In a search for the possible action level of PU, action potentials were recorded, at the same time, from pre- and post-ganglionic fibres (see methods). The recording obtained is illustrated in Fig. 2. The amplitude of the single post-ganglionic potential (II) and of the main component of the pre-ganglionic recording (I) was measured. In all the experiments (five), with 0·18 mM PU, depression of the pre-ganglionic response ranged between 30 and 50 per cent of the depression of the post-ganglionic response.

Effect of PU on synaptic potentials. Figure 3, I, II, III illustrates a typical experiment carried out by recording, as described in the methods. The synaptic potential recorded after curarisation (Fig. 3, II) is depressed promptly and obviously by 0·18 mM PU (III). In seven similar experiments depression to 50 per cent was reached within 42 $\pm$ 2·65 min and recovery upon washing was regularly observed.

Effect of PU on the asynchronous discharge recorded from eserinised ganglia, on (a) pre-ganglionic electrical stimulation, (b) ACh injection. A typical experiment is illustrated in Fig. 4 (a) and (b); both the response evoked by pre-ganglionic electrical stimulation and that evoked by ACh injection are depressed by puromycin to approximately the same extent and for more or less the same time course; this result was consistently obtained in all the experiments (four).

Effect of PU on synthesis and turnover of ACh. The ganglion was incubated for 2 hr in a medium containing [^{3}H]choline (10 μc/ml) and carrier choline (1·5 μg/ml); during this period repetitive activity was induced at 10/sec; then 0·18 mM PU was added. Over the following hour, PU induced the usual depression, to about 50 per

TABLE 3. LABELLED ACh IN GANGLIA INCUBATED WITH (EXPERIMENTAL) AND WITHOUT (CONTROL) PUROMYCIN (EXPERIMENTAL CONDITIONS, IN THE TEXT)

Control (dis./min/ganglion)	Experimental (dis./min/ganglion)	Referred to the control ganglion %	P (%)
64,703 ± 7125 (6)	118,431 ± 14,064 (5)	+83	>99

The values shown are the mean ± S.E.M.; statistical significance of difference is calculated on the basis of Student's *t*-test; number of experiments in brackets.

cent, of the post-ganglionic action potentials evoked by pre-ganglionic stimulation: at the same time, the content of labelled ACh in PU- treated ganglion increased to 183 per cent with respect to the control ganglion, as shown in Table 3.

DISCUSSION

In our previous study of the effects of PU and related compounds on ganglionic function[1], we tentatively ascribed the depression observed to an interaction of PU with (a) some biochemical process (e.g. energy supply and/or ACh system); (b) some neuronal membrane (synaptic and/or extra-synaptic). If these main hypotheses are weighed up, the present investigation provides some evidence for both. For example, the effect of PU on active versus resting ganglion may be higher because it involves (a) some compound which has been exhausted (or accumulated) during sustained activity; (b) some ionic balance or structural state of neuronal membranes which is affected by activity. In fact, the depression of ganglionic response by glucose-lack described by Larrabee and Bronk[15] and Nicolescu *et al.*[7] was markedly reduced and delayed in resting versus active ganglia; the transmission appeared to be selectively affected at the nerve endings while the main metabolic activities of ganglionic neurons were well preserved, along with their electrical response to stimulation with ACh injected into the medium. This response, on the contrary, is depressed by PU to the same extent as that evoked by pre-ganglionic stimuli. This would suggest some post-synaptic effect. On the other hand, a possible impairment of energy-supplying processes in the PU-treated ganglia cannot be excluded. In fact, a 30 per cent inhibition of O_2 consumption by brain tissue in the presence of 0·21 mM PU *in vitro* was reported.[16] Such an inhibition, however, does not necessarily involve a corresponding depression of the post-ganglionic response, as shown by Larrabee *et al.*[17] using sodium azide as a respiratory inhibitor. Another possible interference of PU with energy-supplying processes is represented by the observed acceleration of glycogenolysis in muscle and liver cells treated with PU.[18,19]

A further chemical approach to the PU effect, that concerning its possible interaction with Ca^{2+}, has given a clear indication that this is not the likely mechanism of depression, for the combined evidence from changes of Ca^{2+} and Mg^{2+} levels and from the addition of EDTA (this compound has an effect only at concentrations which are 10–15 times the effective concentrations of PU).

The comparison of action potentials recorded simultaneously from pre- and post-ganglionic trunks suggests the following: first, the pre-ganglionic potentials are de-

pressed by PU to approximately the same extent as observed (with the same concentrations of PU) by Bondeson *et al.*[20] for spinal cord fibres, by Dahl[21] for vagus nerve trunks and by ourselves (unpublished results) for isolated sympathetic nerve trunks. Second, this depression is 30–50 per cent of that displayed at the post-ganglionic level. An explanation of this may be sought among several possible mechanisms. One is the possible amplifying effect of the ganglionic transmission, due to the integrative control of the firing threshold of ganglionic neurons; another is the possible interaction of PU at the synaptic or post-synaptic level. With respect to the latter possibility, we should take into account the depression of the post-ganglionic potentials recorded from curarised ganglia (that is, the electrotonically propagated EPSP of ganglionic neurons): these potentials are depressed to the same extent and for the same time as the action potentials from non-curarized ganglia. In other words, the PU-induced depression reaches its full extent already at the level of the EPSP. Then some other possible mechanism should be considered: (a) a hypothetical curare-like action of PU at the sub-synaptic site; (b) the interaction of PU with post-synaptic membranes (e.g. depolarization); (c) an interference with ACh synthesis, storage and release. A relevant indication is provided by the study of the post-ganglionic discharge elicited by injecting ACh: this stimulation, in providing an excess of transmitter, bypasses any possible effect of PU at the pre-synaptic level. Nevertheless, the discharge thus induced is depressed by PU to the same extent as that induced by supramaximal stimulation of the pre-ganglionic trunk.

The EPSP impairment appears to be due to a disturbance at the post-synaptic level. Besides this likely effect (depolarization of post-synaptic membranes?) an effect on pre-ganglionic nerve endings is indicated by the observed changes in the level of labelled ACh in puromycin-treated ganglia: the 83 per cent increase goes against the hypothesis of an interference with ACh synthesis, but rather suggests an impairment of its release: this by itself could explain most of the observed depression of the EPSP's and of post-ganglionic action potentials. As for the possible mechanism of release impairment, this could be found in the depression of the action potentials of the pre-ganglionic trunk plus some possible action on the potentials of the nerve endings. In fact the latter effect is caused by some drugs through depolarization.

It can therefore be seen that both pre- and post-synaptic effects of PU probably combine in causing the depression of the ganglionic response. Further investigation with proper recording techniques will be necessary to test the main unifying hypothesis (that of some general, and possibly graded, depolarizing action on both pre- and post-synaptic membranes).

Acknowledgement—Thanks are due to Mr. Giulio Berardi for skilful technical assistance.

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