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LONG-TERM POTENTIATION OF SYNAPTIC ACETYLCHOLINE RELEASE IN THE SUPERIOR CERVICAL GANGLION OF THE RAT

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SUMMARY

1. The release of endogenous acetylcholine (ACh) from the *in vitro* rat superior cervical ganglion was measured by assaying the bathing medium. Simultaneously, synaptic transmission in the ganglion was assessed by recording post-ganglionic compound action potentials.

2. A brief period of tetanic preganglionic stimulation (20 Hz for 20 s) induced a long-term potentiation of the post-ganglionic compound action potential.

3. The same tetanic stimulation also consistently induced a long-term potentiation of stimulated ACh release. Spontaneous (non-stimulated) ACh release was not enhanced after tetanic stimulation.

4. The content of ACh in the ganglion was not measurably increased after tetanic stimulation.

5. These results suggest that the long-term increase in synaptic efficacy is due, at least in part, to an increase in the amount of ACh released by the afferent impulse.

INTRODUCTION

Long-term potentiation (l.t.p.) is an hours-long increase in synaptic efficacy that can be induced by merely a few seconds of repetitive synaptic stimulation. L.t.p. has been studied in the hippocampus during the last decade and a variety of mechanisms have been suggested to explain this striking example of synaptic memory (for reviews, see Bliss, 1979; Swanson, Teyler & Thompson, 1982; Lynch & Baudry, 1984; Teyler & Discenna, 1984). Additionally, l.t.p. can be measured in the rat superior cervical ganglion (Brown & McAfee, 1982). Ganglionic l.t.p. results from an increase in the efficacy of nicotinic synaptic transmission without changes in the resting potential or input resistance of the post-synaptic neurone (Briggs, Brown & McAfee, 1983). The potentiation is induced by a brief train of presynaptic stimuli, but does not require activation of the post-synaptic cholinergic receptors during the train (Briggs, Brown & McAfee, 1985). Furthermore, direct activation of the post-ganglionic neurone with a train of non-synaptic stimuli does not cause a potentiation of synaptic transmission. These findings have led us to the idea that ganglionic l.t.p. may result from a

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presynaptic process that induces an hours-long potentiation of evoked acetylcholine (ACh) release. We have tested this hypothesis directly by measuring ACh released from the ganglion under conditions that produce l.t.p. A preliminary report has appeared elsewhere (McCaman, Briggs & McAfee, 1984).

METHODS

Superior cervical ganglia were quickly removed from Sprague–Dawley rats (either sex, 170–220 g) which had been sacrificed by decapitation. The dissection and desheathing were as previously described (McAfee, 1982). The tissue was maintained *in vitro* at ambient temperature (21–23 °C) by superfusion with media based on Locke solution of the following composition (mM): NaCl, 136; KCl, 5·6; $CaCl_2$, 2·2; $MgCl_2$, 1·2; NaH_2PO_4, 1·2; $NaHCO_3$, 20; and dextrose, 8. The media were equilibrated with 95 % O_2–5 % CO_2, and had a pH of 7·2. Choline chloride (10 μM) was added to the bathing medium in the experiments where the ganglionic content of ACh was measured. The medium was then comparable to that used by other laboratories performing similar experiments. Choline was not added to the medium when only the release of ACh was measured. Nevertheless, both ACh release and ganglionic transmission were well maintained during stimulation for 2 h.

ACh content in the ganglia

The ganglia were isolated and maintained as homologous pairs and, after being desheathed, were incubated without stimulation for a minimum of 60 min before further treatment. In some experiments the preganglionic nerve of only one member of each pair was repetitively stimulated with supramaximal monophasic current pulses (500 μs duration). All ganglia were finally homogenized in 50 μl 0·1 N-HCl and stored at −20 °C (3 h to 3 days) for subsequent determination of endogenous ACh.

ACh release from the ganglion

The ganglion was placed into a small-volume experimental chamber which minimized the dilution of the ACh released from the ganglion. The chamber was fashioned from a 1 ml plastic pipette by removing a portion of the wall between two adjacent 0·1 ml graduation marks. The total volume of this chamber, with the ganglion and electrodes installed, was about 65 μl.

The preganglionic nerve was stimulated with one suction electrode, using monophasic current pulses (500 μs duration) at an intensity 1·5-fold greater than that needed to generate a maximal post-ganglionic response. The post-ganglionic compound action potential, recorded from the internal carotid nerve with a second suction electrode, was displayed on an oscilloscope and digitized by a computerized data-acquisition system.

The Locke bathing medium contained the anticholinergic compounds atropine (2 μM) and either *d*-tubocurarine (100 μM) or hexamethonium (200 μM). These substances were used to block muscarinic transmission and to partially inhibit nicotinic transmission so that the post-ganglionic response was submaximal before the induction of l.t.p. (cf. Briggs *et al.* 1985). The medium also contained 20 μM-physostigmine to block hydrolysis of the released ACh. Preliminary experiments showed that 20 μM-physostigmine inhibited cholinesterase in the ganglion sufficiently to allow 85 % recovery of 5 pmol authentic ACh added to the bathing medium for a 10 min incubation.

Ganglia were superfused for 2 h before samples of the bathing medium were collected for the assay of ACh. During the sample collection period, superfusion was stopped and the bath was oxygenated by gently bubbling 95 % O_2–5 % CO_2 through a 27 gauge needle cemented into a side wall. Samples containing the released ACh were obtained by withdrawing 50 μl (about 80 % total volume) of the bathing medium and replacing it with 50 μl fresh, oxygenated medium. The samples were immediately frozen and could be stored at −20 °C overnight with no detectable loss of ACh, or for 3 days with a 5–10 % loss.

ACh release was measured in the absence of stimulation (spontaneous release) and during low-frequency stimulation at 0·2 Hz. These measures were made before and after tetanic preganglionic stimulation at 20 Hz for 20 s. The release of ACh during 0·2 Hz stimulation was allowed to stabilize for 25 min before measurements were begun.

Preparation of samples for assay

ACh in the homogenates and the superfusates of the ganglia was extracted and assayed using the procedures described in detail by Goldberg & McCaman (1973, 1974) and modified by McCaman & Stetzler (1977). These procedures, as summarized below, were necessary to prevent salts and unknown substances from interfering with the assay for ACh.

The frozen homogenates of the ganglia (in 0·1 N-HCl, 50 μl) were thawed, re-homogenized and centrifuged at 1000 *g* for 15 min (2 °C). Replicate 8 μl portions of the supernatant were placed in clean microtubes, dried in a vacuum centrifuge, resuspended in 30 μl sodium phosphate buffer (pH 7·5), and then further processed as described below. The pellet resulting from centrifugation of the homogenate was redissolved in 0·5 M-NaOH and assayed for protein (Lowry, Rosebrough, Farr & Randall, 1951). The superfusates of the ganglia were extracted with an equal volume of chloroform to remove the anticholinesterase (physostigmine) that would otherwise interfere with the radioenzymatic assay for ACh. A 45 μl portion of the upper, aqueous layer was removed and placed in a clean microtube.

The ACh and choline present in the samples derived from the homogenates and the superfusates were extracted into 40 μl 3-heptanone containing sodium tetraphenylboron (5 mg/ml). After mixing and brief centrifugation, 33 μl of the upper organic layer (containing the ACh and choline) was removed and placed in another microtube containing 28 μl 0·5 N-HCl. After mixing and brief centrifugation, the upper organic layer was aspirated off and discarded. Finally, a 22 μl portion of the remaining aqueous layer was removed, placed in a clean tube, and taken to dryness in a vacuum centrifuge (Speed Vac Concentrator, Savant Instrument Co., Hicksville, NY, U.S.A.). The dried samples (including standards and blanks) were analysed for their content of ACh on the same or the next day, but they could be stored for several weeks at −20 °C or for several months at −70 °C.

ACh assay

The extracted and dried samples received 5 μl of an incubation mixture consisting of the following subtances at their final concentrations: sodium phosphate buffer, 0·15 M, pH 8·5; ATP (pH 7·0), 400 μM; $MgCl_2$, 6 mM; dithiothreitol, 0·3 mM; and choline kinase, approximately 4·5 μg/μl. After incubation for 20 min at 37 °C, the tubes were returned to an ice bath (0–2 °C), and 5 μl of a second incubation mixture consisting of phosphate buffer, acetylcholinesterase, and [γ-^{32}P]ATP was added. The composition of this last incubation mixture and all other aspects of the assay were as described by McCaman & Stetzler (1977).

Standards of authentic ACh and choline were added to the media used to homogenize or superfuse the ganglia, correcting for losses in the extraction procedure. The amount of ACh in the original sample which had twice the radioactivity of the reagent blank was 0·05–0·10 pmol.

Endogenous choline was released from the ganglion at the rate of 1 pmol/min. This choline had little effect on the measurement of ACh release because 99·5% of the choline is excluded by the assay procedures.

Materials

The materials and their sources were: choline chloride (Matheson, Coleman and Bell Manufacturing Chemists, Norwood, OH, U.S.A.); acetylcholine bromide, acetylcholinesterase (Type V, lyophilized salt-free, from electric eel), adenosine 5′-triphosphate (ATP, disodium salt, from equine muscle), atropine sulphate, choline kinase, hexamethonium bromide, physostigmine sulphate, and *d*-tubocurarine chloride (Sigma Chemical Co., St. Louis, MO, U.S.A.); and [γ-^{32}P]ATP (35 Ci/mmol, New England Nuclear, Boston, MA, U.S.A.).

RESULTS

ACh release evoked from nerve terminals in the ganglion was estimated by measuring ACh levels in samples of the bathing medium during preganglionic stimulation at a low frequency (0·2 Hz). The efficacy of synaptic transmission was estimated at the same time from records of the post-ganglionic compound action

potential. Fig. 1 demonstrates that a brief period of tetanic stimulation induced a long-term potentiation (l.t.p.) of the post-ganglionic compound action potential and, at the same time, a long-term potentiation of ACh release during the 0·2 Hz test stimulation.

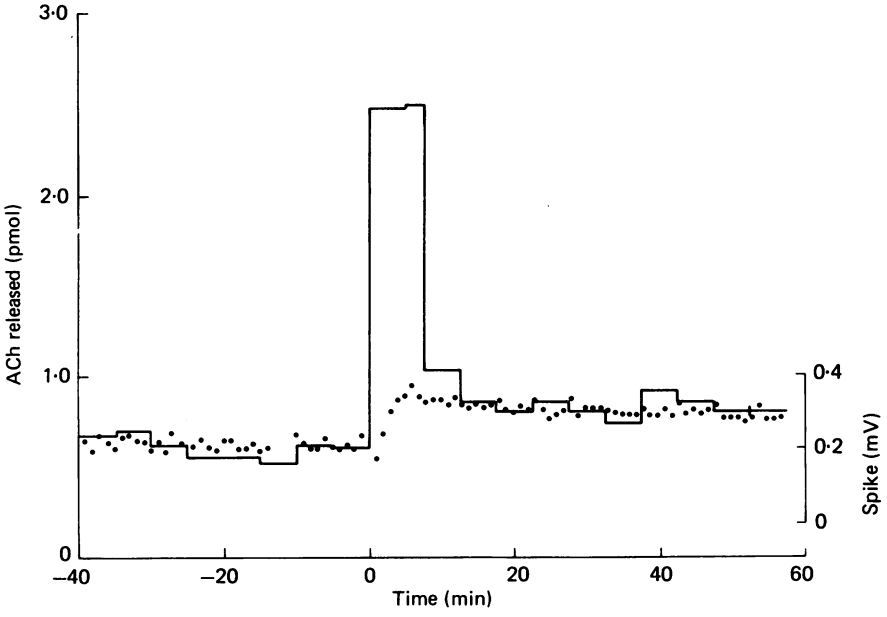

Fig. 1. A long-term potentiation of ganglionic transmission and of acetylcholine release. All data are from one ganglion. The ganglion was bathed in a medium containing atropine (2 μM), *d*-tubocurarine (100 μM) and physostigmine (20 μM). The preganglionic nerve was stimulated at 0·2 Hz except during tetanic stimulation (20 Hz for 20 s at 0 min). Ganglionic transmission was measured by recording the amplitude of the post-ganglionic compound action potential spike (filled circles). At the same time, samples of the bathing medium were collected and the efflux of endogenous ACh was measured by assaying the amount of ACh released into the bathing medium (continuous line). The bathing medium was exchanged five times between 5 and 8 min after tetanic stimulation in order to ensure complete exchange of acetylcholine. Otherwise, samples were collected once every 5 min. During tetanic stimulation, there was a large increase in the efflux of ACh; this ACh was washed out in 12 min (see also Fig. 2). After tetanic stimulation, there was a prolonged potentiation of ganglionic transmission and of ACh efflux. Both measures were significantly greater between 12 and 60 min after tetanic stimulation compared to before tetanic stimulation ($P < 0·001$, two-tailed unpaired t test). Similar results were obtained in six other experiments.

Spontaneous (non-stimulated) ACh release was not potentiated after similar tetanic stimulation (Fig. 2). The induction of l.t.p. by this tetanic stimulation could be confirmed by measuring the post-ganglionic response to three single preganglionic stimuli (1/13 min) before and after tetanic stimulation. These few stimuli added an insignificant amount of ACh to that spontaneously released. Spontaneous ACh release also was not potentiated if the tetanic stimulation was preceded by stimulation at 0·2 Hz for 60 min (not shown).

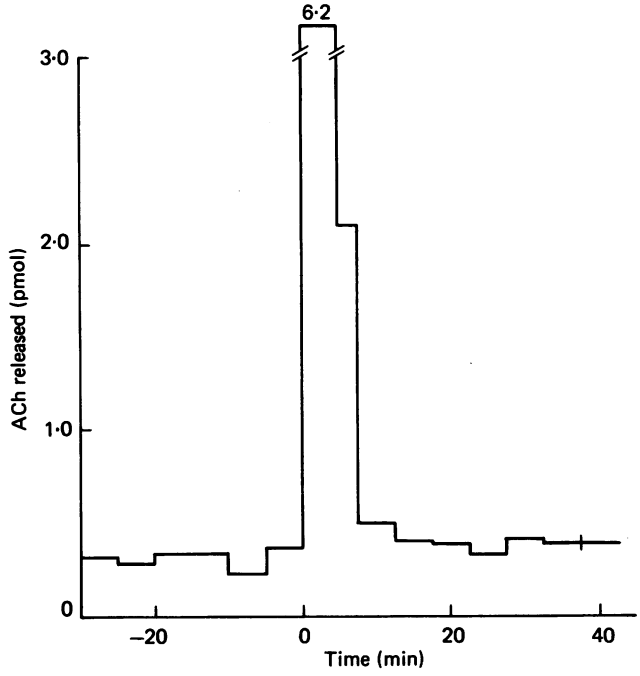

Fig. 2. Spontaneous ACh release before and after tetanic stimulation. The experimental conditions were similar to that of Fig. 1, except that the preganglionic nerve was stimulated only tetanically (20 Hz for 20 s at 0 min) and with three single test stimuli (1/13 min) before and after tetanization. The tetanic stimulation caused a large increase in ACh efflux, peaking at 6·2 pmol. The ACh released during tetanic stimulation was washed out in 12 min. L.t.p. of ganglionic transmission was induced (not shown), but there was little or no effect on the spontaneous release of ACh measured 12 min and more after the tetanic stimulation. This Figure shows the greatest difference observed in spontaneous release after tetanic stimulation compared to before tetanic stimulation (six experiments).

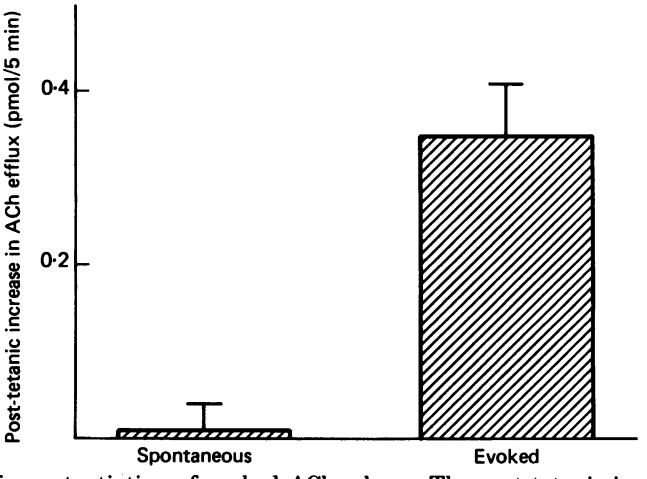

Fig. 3. Selective potentiation of evoked ACh release. The post-tetanic increase in ACh efflux was computed in each experiment by subtracting the mean ACh efflux 40–0 minutes before tetanic stimulation from the mean ACh efflux 12–60 min after tetanic stimulation (20 Hz for 20 s). The data are shown as mean ± S.E. of mean from six experiments measuring spontaneous release and seven experiments measuring evoked release.

The evoked release of ACh, but not the spontaneous release of ACh, correlated with l.t.p. in the ganglion. Fig. 3 shows the increased amount of ACh released 12–60 min after tetanic stimulation (20 Hz for 20 s). Evoked ACh release was increased for at least 1 h after tetanic stimulation in each of the seven experiments. This potentiation of evoked release averaged $41 \pm 7\%$ (mean $\pm$ S.E. of mean). The post-ganglionic compound action potentials were potentiated by $54 \pm 11\%$, 30 min after the same tetani. In contrast, there was little or no increase in spontaneous release ($n = 6$) even though the compound action potential was potentiated by $51 \pm 6\%$, 30 min after the tetani ($n = 4$).

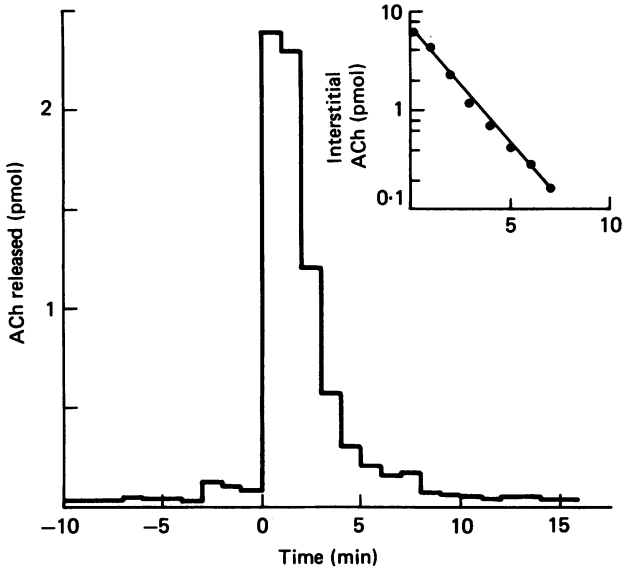

Fig. 4. Efflux of ACh released during tetanic stimulation. The preganglionic nerve was stimulated tetanically (20 Hz for 20 s at 0 min) but otherwise was not stimulated. Samples of the bathing medium were collected once every minute. The brief tetanic stimulation caused a large increase in ACh efflux which returned to the non-stimulated level in 10 min. These data were used to compute the total amount of ACh released by tetanic stimulation (7·0 pmol), and the amount of tetanically released ACh remaining in the tissue at various times after stimulation. The latter is shown on a logarithmic ordinate as a function of time (inset). In this experiment, ACh cleared the ganglion with a half-decay time of 1·26 min.

In previous experiments, we normally measured l.t.p. with a test stimulation frequency of 0·017 Hz (1/min; Briggs *et al.* 1985). Under these conditions, the initial potentiation was $81 \pm 13\%$ and the time constant of decay was 31 ± 5 min ($n = 4$). In the present experiments, the testing frequency was increased to 0·2 Hz so that the release of ACh during stimulation was at least 3-fold greater than the non-stimulated release. Under these conditions, the initial potentiation was $44 \pm 8\%$ and the time constant of decay was 170 ± 60 min ($n = 4$). However, the magnitude 30 min after tetanic stimulation was independent of the testing frequency even when it was decreased to 1/13 min.

The post-tetanic increase in ACh release was not due to residual efflux of the ACh

released during tetanic stimulation. Only the evoked release of ACh, not the spontaneous release, showed such an increase (Fig. 3). Additionally, in three experiments we measured the rate at which ACh cleared the ganglion; an example is shown in Fig. 4. Tetanic preganglionic stimulation (20 Hz, 20 s) released a total of 7 ± 1 pmol ACh. This ACh cleared the ganglion and appeared in the bathing medium according to a single-compartment model with a half-decay time of $1{\cdot}2 \pm 0{\cdot}05$ min. Within 10 min after the tetani, the efflux of ACh was indistinguishable from the base-line spontaneous efflux. Thus, the hour-long post-tetanic increase in evoked ACh efflux is apparently due to a presynaptic mechanism that enhances the release of ACh from stimulated nerve terminals.

TABLE 1. Repetitive stimulation and ACh content in the rat ganglion

	ACh content (pmol/μg protein)			
	Non-stimulated ganglia			
	A	B	Stimulated ganglia	Paired difference
Untreated	$1{\cdot}8 \pm 0{\cdot}2$	$1{\cdot}7 \pm 0{\cdot}1$	—	$-0{\cdot}1 \pm 0{\cdot}1$
20 Hz for 20 s	$1{\cdot}3 \pm 0{\cdot}1$	—	$1{\cdot}5 \pm 0{\cdot}1$	$0{\cdot}2 \pm 0{\cdot}1$
20 Hz for 60 min	$1{\cdot}4 \pm 0{\cdot}2$	—	$1{\cdot}4 \pm 0{\cdot}1$	$0{\cdot}01 \pm 0{\cdot}20$

In the untreated group, no ganglion was stimulated and the individual members of each homologous pair of ganglia were assigned to subgroup A or B. The paired difference shown was computed by subtracting the ACh content of ganglion B from that of ganglion A. In the other groups, one member of each pair of ganglia was stimulated and then allowed to rest for 5 min after stimulation for 20 s, or for 17 min after stimulation for 60 min. The other member of each pair was the non-stimulated control. Data are shown as mean $\pm$ S.E. of mean ($n = 6$ for each group). Tetanic stimulation did not induce a statistically significant increase in acetylcholine content.

Prolonged (1 h) stimulation of feline sympathetic ganglia at 20 Hz has also been shown to induce an increase in the evoked release of ACh (Birks, 1977, 1978; Collier, Kwok & Welner, 1983). This prolonged tetanic stimulation also produced an increase in the ACh *content* of feline ganglia. However, we observed no detectable increase in the content of ACh when one member of each homologous pair of rat ganglia was stimulated at 20 Hz for 20 s and then rested for 5 min, a treatment that always induces l.t.p. in the rat ganglion (Table 1). Even stimulation at 20 Hz for 60 min, followed by 17 min rest, did not significantly increase the content of ACh in the rat ganglia.

DISCUSSION

The phenomenon of l.t.p. is familiar from many studies in the hippocampus, but the mechanisms by which hippocampal l.t.p. is induced and expressed are not yet well understood. Increased release of radiolabelled aspartate (Skrede & Malthe-Sorenssen, 1981) and glutamate (Dolphin, Errington & Bliss, 1982) has been related to l.t.p., suggesting that the phenomenon is presynaptic. On the other hand, evidence for an increase in *in vitro* glutamate binding has also been presented and this has been

proposed to represent a purely post-synaptic mechanism for l.t.p. (Lynch & Baudry, 1984).

In the sympathetic ganglion, ACh is the major neurotransmitter and post-synaptic discharge is induced almost exclusively by activation of nicotinic cholinergic receptors. Our previous studies demonstrated that l.t.p. in the rat superior cervical sympathetic ganglion results from an increase in the efficacy of nicotinic transmission (Brown & McAfee, 1982; Briggs *et al.* 1983; Briggs *et al.* 1985). This can be measured intracellularly by recording the nicotinic synaptic depolarization, or extracellularly by recording the post-ganglionic compound action potential. The present studies indicate that the evoked release of ganglionic ACh is potentiated for at least 1 h after a brief, high-frequency stimulus train (20 Hz for 20 s). This long-term potentiation of the evoked release of ACh is accompanied by a long-term potentiation of the post-ganglionic compound action potential. The quantitative relationship between the compound action potential and the amount of acetylcholine released is unknown, but is likely to be non-linear.

It is reasonable to assume that most of the evoked ACh release originated from the cholinergic nerve terminals making nicotinic synaptic contact with post-ganglionic neurones. Thus, l.t.p. in the rat superior cervical ganglion appears to arise, at least in part, from a presynaptic mechanism that produces an hours-long potentiation of evoked ACh release. Similarly, quantal analysis of l.t.p. in the crayfish neuromuscular junction has also indicated that the locus of l.t.p. is presynaptic (Baxter, Bittner & Brown, 1985).

In the sympathetic ganglion of the frog, repetitive stimulation has also been found to induce an hours-long increase of the nicotinic synaptic response, but apparently by a post-synaptic mechanism (Kumamoto & Kuba, 1983). However, this effect appears to differ from l.t.p. in the rat superior cervical ganglion in other aspects. In the frog ganglion, the potentiation of synaptic transmission was induced antidromically by repetitive stimulation of the post-ganglionic nerve. In the rat ganglion, repetitive non-synaptic stimulation of the post-ganglionic neurone did not induce l.t.p. (Brown & McAfee, 1982; Briggs *et al.* 1983) and neither cholinergic nor adrenergic transmission appears to be responsible for the induction of l.t.p. by repetitive synaptic stimulation (Briggs *et al.* 1985).

A potentiation of transmitter release could result from increased efficacy of stimulus–secretion coupling mechanisms. For example, there may be an increased ability of the action potential to depolarize the terminal, an increase of Ca^{2+} channel opening, or a potentiation of the vesicle–membrane fusion processes. Alternatively, an augmentation of release could result from an augmentation of transmitter content in the nerve terminal.

The latter hypothesis is testable in the superior cervical ganglion because 85% of the ganglionic ACh is releasable by stimulation and is therefore probably contained in the afferent cholinergic nerve terminals (MacIntosh & Collier, 1976). In the feline superior cervical ganglion, stimulation for 1 h or more at 20 Hz causes, after a 15 min delay, an increase in the content of ACh (rebound ACh) and an increase in the release of ACh (Birks, 1977, 1978; Collier *et al.* 1983). However, under our conditions, stimulation of the rat superior cervical ganglion at 20 Hz for 20 s (which always causes l.t.p.) or at 20 Hz for 60 min did not elicit a measurable increase in the ganglionic

content of ACh. Thus, the long-term potentiation of ACh release from the rat superior cervical ganglion does not seem to be secondary to an increase in the content of ACh. The ability of elevated K^+ (50 mM for 3 min) to induce a potentiation resembling l.t.p. (Briggs *et al.* 1985) and the inability of elevated K^+ (46 mM for 60 min) to induce rebound ACh (Collier *et al.* 1983) appears to differentiate further the phenomena.

While our experiments were performed at 22 °C, other investigators have also failed to detect rebound ACh in the rat superior cervical ganglion at 37 °C (Sacchi, Consolo, Peri, Prigioni, Ladinsky & Perri, 1978). In our studies and those of Sacchi *et al.*, the rat superior cervical ganglion was maintained *in vitro* with superfusion of the oxygenated bathing medium. However, in the studies of rebound ACh in the feline superior cervical ganglion, the tissue was maintained *in situ* with intact circulation or with perfusion through the arterial supply. Thus, the discrepancy concerning the formation of rebound ACh may be related to the species of animal studied or to the conditions under which the tissue was maintained.

Our studies of ACh release suggest that a presynaptic mechanism contributes to the long-term potentiation of nicotinic synaptic transmission in the superior cervical ganglion. However, these results do not rule out the possibility that some post-synaptic mechanism also contributes to l.t.p. There could be an accompanying increase in post-synaptic nicotinic receptors, or a modulation of an active post-synaptic conductance. Indeed, a single type of conductance modulation, if localized post-synaptically as well as presynaptically, could increase active post-synaptic depolarization and excitability as well as increase transmitter release. Complementary approaches, such as quantal analysis, would be useful in resolving these issues.

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