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Extracellular calcium alters frequency modulation of [^{3}H]acetylcholine release from rat hippocampal slices

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The concentration of extracellular Ca^{2+} has been shown to enhance or attenuate [^{3}H]acetylcholine (ACh) release subsequent to a conditioning stimulus in rat brain hippocampal slices. Slices were incubated in vitro in [^{3}H]choline solution. Subsequently the slices were subjected to two consecutive electrical stimulations separated by 15 or 30 min at 0.25, 1, 4 and 16 Hz and [^{3}H]ACh release was assessed. It was found that a conditioning stimulus may reduce [^{3}H]ACh release during a second stimulation. This phenomenon is frequency related and disappears when the two stimulations are 30 min apart. High extracellular Ca^{2+} (4.0 mM) further attenuated [^{3}H]ACh release during the second stimulation, whereas low Ca^{2+} (0.32 mM) abolished the decrease in [^{3}H]ACh release following the second stimulation in all frequencies tested.

Release of acetylcholine (ACh) in the central and peripheral nervous system is controlled by a negative feedback loop mediated by presynaptic muscarinic receptors. Several groups of researchers have demonstrated a negative relationship between stimulation frequency and [^{3}H]ACh release[1,3,7]. [^{3}H]ACh release is attenuated following a first train of electrical stimuli depending upon the frequency of stimulation and the interval between trains. The role of extracellular Ca^{2+} in this phenomenon was examined in the present report.

Tritiated choline ([^{3}H]Ch, spec. act. 80 Ci/mmol) was purchased from New England Nuclear (Boston, MA 02118). ACS scintillation fluid and NCS tissue solubilizer were supplied by Amersham (Arlington Heights, IL 60005). Hemicholinium (HC-3) and tetrodotoxin were purchased from Sigma (St. Louis, MO 63178). All other chemicals were of analytical grade. Male Harlan Sprague–Dawley outbred rats (150–250 g) from Harlan Sprague Dawley Inc. (Indianapolis, IN 46229) were used for all experiments. Rats were killed by decapitation. Their brains were removed and placed in an oxygenated cold (3–5 °C) preparation buffer. The hippocampi were dissected and transverse slices (400 μm) were prepared using a McIlwain tissue chopper. Slices from the medial section of the hippocampus were incubated for 30 min in 5 ml of preparation buffer containing 20 μCi of [^{3}H]Ch. The composition of the buffer was as follows (mM): NaCl 124, KCl 31, $NaHCO_3$ 25.6, $CaCl_2$ 1.3, $MgSO_4$ 1.3, KH_2PO_4 1.3, glucose 10. In some experiments the Ca^{2+} concentration was altered from 0.32, 1.3 and 4.0 mM. Both osmolarity and ionic strength of the modified buffer were adjusted with glucose and NaCl. After incubation the slices were washed, placed in the chambers and superfused for 40 min with a flow rate of 1 ml/min. The perfusate contained HC-3 to prevent reuptake of [^{3}H]Ch and was constantly equilibrated with 5% CO_2–95% O_2. All solutions and slices were held at 35 °C. After 40 min of superfusion, the fluid was collected at 5-min intervals. At 50 min and at 65 or 80 min the slices were stimulated with 25 V rectangular electrical pulses (20 mA peak), each 2 ms in duration. Stimulation frequency ranged from 0.25 to 16 Hz. One milliliter of each collected perfusate was removed and added to

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ACS. At the termination of the experiment, the slices were dissolved in 100 μl NCS and counted in ACS. Total disintegrations per minute were determined using a Beckman programmed LS 5801 series liquid scintillation spectrometer.

Each evoked release was expressed as a percentage of the pre-existing pool of [^{3}H]ACh present at the beginning of the stimulation. The ratio of the fraction released during the second stimulation (S2) over the fraction released during the first stimulation (S1) was also considered in the analysis.

Frequency relationship and [^{3}H]ACh release. The fraction of tissue [^{3}H]ACh content released during the first stimulation (S1) was used to analyze the frequency and [^{3}H]ACh release relationships. Fig. 1 shows the fraction of the pool released per one pulse as a function of frequency. Release was seen to decrease sharply with increasing frequencies.

The ratio of S2/S1 [^{3}H]ACh release also was found to decrease as the frequency of stimulation increased, as shown in Fig. 2. Two-way analysis of variance revealed significant differences in overall frequency related release. Subsequent paired *t*-tests demonstrated S2 to be significantly lower at the stimulation frequencies of 4 and 16 Hz, but not for 0.25 and 1 Hz (Fig. 2). All frequencies were tested with 2 min of stimulation time resulting in a frequency related increase in the number of pulses delivered.

In order to determine whether these differences could be related to neurotransmitter pool depletion, 120 pulses at 16 Hz over 7.5 s were applied. This also

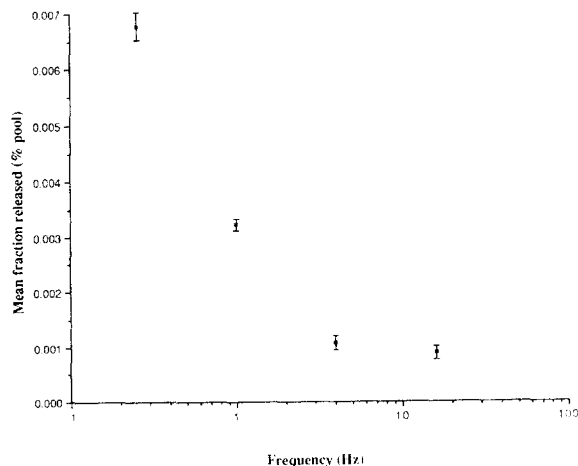

Fig. 1. Mean fraction ± S.E.M. of [^{3}H]ACh released per impulse (as a % of the pool) in the presence of 1.3 mM Ca^{2+}.

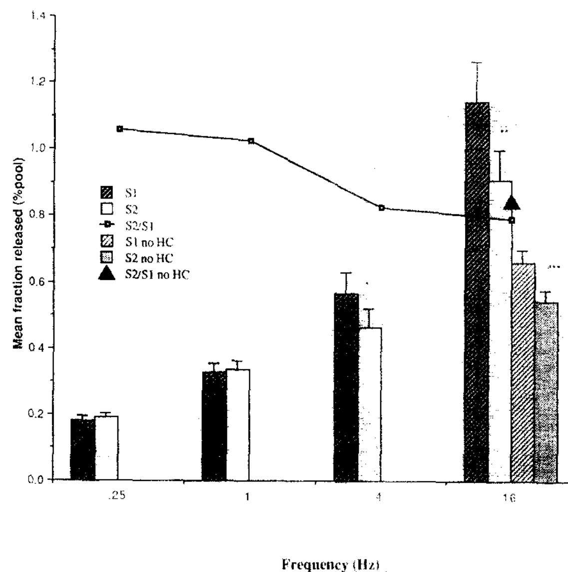

Fig. 2. Mean fraction ± S.E.M. of [^{3}H]ACh released (as a % of the pool) during a 2-min stimulation period in the presence of 1.3 mM Ca^{2+}. The mean fraction released at different frequencies is plotted as a pair of vertical bars to indicate the amount of [^{3}H]ACh released during S1 and S2. The ratio S2/S1 is plotted as a line. As illustrated for some slices stimulated at 16 Hz, HC-3 was omitted with no qualitative difference in the results. Each mean is based on 8–19 observations. $*P < 0.05$; $**P < 0.005$; $***P < 0.01$ determined by paired *t*-test.

resulted in a significantly lowered [^{3}H]ACh release during S2. However, the same number of pulses applied at 1 Hz for 2 min did not lower S2. In addition the S1 mean fraction ± S.E.M. released during 120 pulses applied at 16 Hz was smaller than the mean fraction released during application of 120 pulses at 1 Hz (0.121 ± 0.027 vs 0.327 ± 0.008).

To assess the possibility that the use of HC-3 may interfere with the rate of ACh synthesis[8], we performed an additional experiment omitting that agent from the perfusing buffer. The slices in that experiment were stimulated twice for 2 min at a frequency of 16 Hz. As expected, the tritium (^{3}H) overflow was significantly reduced, but the ratio of fractional release during both stimulations remained the same (Fig. 2). Additionally, the release of [^{3}H]ACh during S2 was significantly smaller than during S1.

The duration of this effect was studied by increasing the time interval between stimuli. Stimuli applied at 16 Hz (120 pulses) 15 min apart resulted in a significant decrease in [^{3}H]ACh released during S2. However, 16 Hz stimulation (120 pulses) separated by 30 min did not decrease [^{3}H]ACh release during

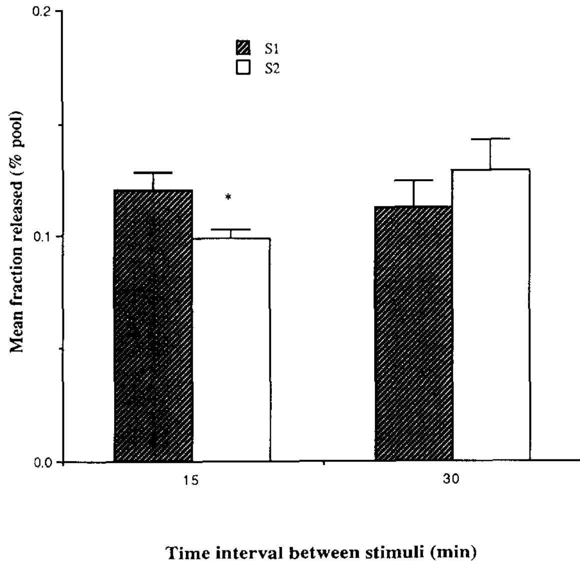

Fig. 3. Mean fraction ± S.E.M. of [^{3}H]ACh released (as a % of the pool) during a 7.5-s stimulation period with 16 Hz (120 pulses) in the presence of 1.3 mM Ca^{2+}. S1 and S2 stimuli were 15 and 30 min apart. Each mean is based on 10–19 observations. *$P < 0.01$ determined by paired t-test.

S2 (Fig. 3). Thus the reduced release of [^{3}H]ACh with high frequencies is reversible.

The same pattern of [^{3}H]ACh was observed in the presence of 1 μM tetrodotoxin (Table I). In this case slices were perfused with buffer containing 1.3 mM $CaCl_2$ and tetrodotoxin throughout the experiment. Slices were stimulated for 2 min at 4 and 16 Hz.

The Ca^{2+} channel (L type) activator[4], BAY K 8644, did not influence the frequency related attenuation of [^{3}H]ACh release (Table I).

Effects of low and high extracellular Ca^{2+} on [^{3}H]ACh release. A Ca^{2+} concentration of 0.13 mM decreased the stimulated release of [^{3}H]ACh to undetectable levels. The Ca^{2+} concentration of 0.39 mM was high enough to support the stimulated release of [^{3}H]ACh at frequencies of 16 and 4 but not at 1 and 0.25 Hz. At this low Ca^{2+} concentration stimulation at 4 and 16 Hz for 2 min did not lower the fraction released during subsequent stimulation (Fig. 4). In the presence of 4 mM $CaCl_2$ the stimulation at all frequencies resulted in attenuated release during S2 (Fig. 5).

Our study shows a steep negative relationship between ACh release and frequency of stimulation. This observation is in agreement with previous reports[1,3,7]. In addition, the frequency of the first stimulation can lower the release evoked by the second stimulation. The phenomenon seems to be restricted to nerve endings since it was observed in the presence of 1 μM tetrodotoxin. It may be worth noting that in our experimental model, [^{3}H]ACh release is evoked

TABLE I

The effect of tetrodotoxin and BAY K 8644 on electrically stimulated [^{3}H]ACh release

Both substances were present in the perfusion buffer during S1 and S2 stimulation trains. Note the lack of effect of either of these substances on the described phenomenon. The differences in S1 release in the presence of two concentrations of BAY K 8644 do not differ.

Agent concentration	*Frequency (Hz)*	*Mean release S1 ± S.E.M. (% of pool)*	*Mean release S2 ± S.E.M. (% of pool)*
Tetrodotoxin	4	0.062 ± 0.012	0.037 ± 0.009*
1 μM	16	0.209 ± 0.059	0.147 ± 0.038*
BAY K 8644			
100 nM	16	1.082 ± 0.039	0.866 ± 0.042*
1 μM	16	0.983 ± 0.032	0.836 ± 0.044*

*$P < 0.05$ determined by paired t-test.

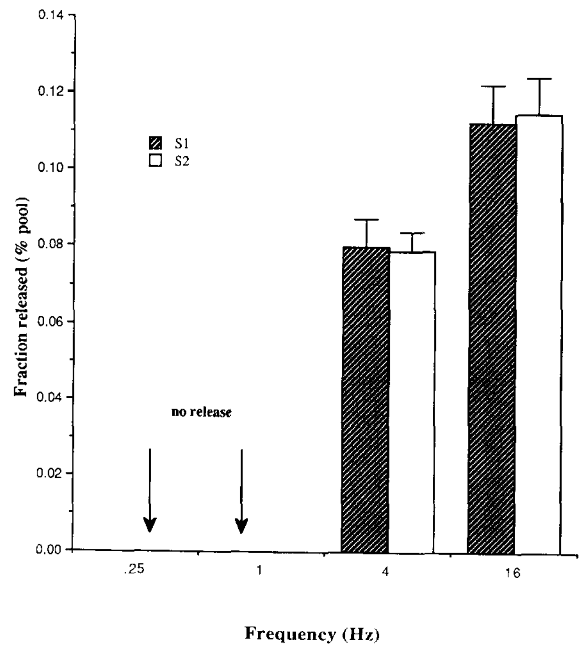

Fig. 4. Mean fraction ± S.E.M. of [^{3}H]ACh released (as a % of the pool) during a 2-min stimulation period in the presence of 0.32 mM Ca^{2+}. At the lower frequencies no release of [^{3}H]ACh was observed. At the higher frequencies [^{3}H]ACh release did not differ during the S1 and S2 stimulation periods. Each mean is based on 10 observations.

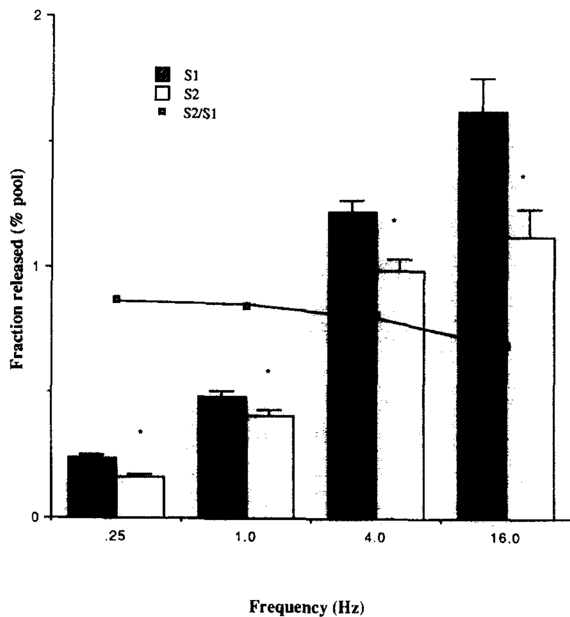

Fig. 5. Mean fraction ± S.E.M. of [^{3}H]ACh released (as a % of the pool) during a 2-min stimulation period in the presence of 4.0 mM Ca^{2+}. The vertical bars represent the amount of [^{3}H]ACh released (as a % of the pool) during a 2-min stimulation period. The ratio S2/S1 is plotted as a line. Each mean is based on 5 observations; $*P < 0.01$ determined by paired *t*-test.

by stimulation of both axons and nerve endings because in the presence of tetrodotoxin the mean fraction of neurotransmitter released was smaller than without it. It appears that the lowered release of [^{3}H]ACh during the second stimulation does not depend on significant depletion of the neurotransmitter pool. The observed levels of ACh in vitro are usually 4–10 times greater than levels measured in the same brain areas after fast microwave fixation[9]. Since the synthesis rate in slices also is high the possibility of a significant depletion of acetylCoA in the slice may be excluded. Our results with controlled number of pulses delivered at different frequencies and the results without HC-3 in the buffer suggest that the mechanism of the observed phenomenon is not due to ACh synthesis alteration, but rather other events triggered by the frequency of stimulation. The phenomenon observed is frequency dependent since it can be elicited by 120 pulses depending on the frequency of stimulation. It is also Ca^{2+} dependent. At a Ca^{2+} concentration of 1.3 mM, higher frequencies (4 and 16 Hz) significantly attenuated [^{3}H]ACh release during the second stimulation. A low Ca^{2+} concentration (0.32 mM) blocked this effect, while a high Ca^{2+} concentration (4.0 mM) enhanced the effect beginning with the lowest frequency tested (0.25 Hz). Although the attenuation of [^{3}H]ACh release can be controlled by the extracellular Ca^{2+} concentration, the L-calcium channel activator, BAY K 8644 was without effect. This observation is consistent with the study of Starke et al.[6], who showed that ACh release is not affected by Ca^{2+} channel blockers and with the report on the postsynaptic localization of L-type Ca^{2+} channels[5]. However, Woodward and Leslie[10] reported an effect of BAY K 8644 on release of dopamine which may indicate an unequal distribution of L-type Ca^{2+} channels in different brain structures.

In summary:

(1) The modification of [^{3}H]ACh release develops in less then 7.5 s and lasts less than 30 min.

(2) It appears to be dependent on the extracellular Ca^{2+} concentration. It is unlikely that it involves L-type Ca^{2+} channels.

(3) The rate of Ca^{2+} concentration increase inside the cell may play a role in the development of this phenomenon.

It has been reported[2] that changes of extracellular Ca^{2+} concentration affect catecholamine secretion parallel to the uptake of Ca^{2+} in cultured chromaffin tissue. This suggests that the results presented here are due to involvement of Ca^{2+} channels present on cholinergic nerve terminals.

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