

Research Reports

Quantitative correlation between tetanus-induced decreases in extracellular calcium and LTP

Bert P.C. Melchers*, Cyriel M.A. Pennartz, Wytse J. Wadman and
Fernando H. Lopes da Silva

Department of General Zoology, University of Amsterdam, Amsterdam (The Netherlands)

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Decreases in the extracellular calcium concentration ($[Ca^{2+}]_o$), induced by tetanization of the Schaffer collaterals in rat hippocampal slices, were measured by means of Ca^{2+} -sensitive microelectrodes. The amount of long term potentiation (LTP) of the evoked field potentials, induced by this tetanus, was determined. A positive correlation was found between the amplitude of the tetanus induced decrease in $[Ca^{2+}]_o$ and the amount of LTP that was elicited. The *N*-methyl-D-aspartate (NMDA) receptor antagonist 2-amino-phosphonovalerate decreased both the tetanus-induced decreases in $[Ca^{2+}]_o$ and the amount of LTP that was induced. We conclude that the amount of Ca^{2+} that enters the cell during a tetanus is of major importance in the induction process of LTP.

INTRODUCTION

Tetanic stimulation of afferents, for instance, in the hippocampus can lead to a long-lasting facilitation of synaptic transmission. This has been termed long-term potentiation (LTP)³. The mechanism underlying this form of synaptic plasticity is not fully understood but evidence has been accumulating that the intracellular Ca^{2+} concentration plays an important role in the induction of the phenomenon: LTP can only be induced if Ca^{2+} is present in the extracellular medium, both during^{7,13} and shortly after the tetanus¹³, and a shortlasting elevation of the extracellular calcium concentration can induce LTP^{22,28}. During tetanization the intracellular Ca^{2+} concentration does increase^{15,16} and Baimbridge and Miller² have shown an increased uptake and retention of Ca^{2+} in slices in which LTP was elicited. Furthermore Lynch et al.¹⁸ have shown that injection of a Ca^{2+} -chelating agent (EGTA) prevents LTP in the post-

synaptic neuron.

A point of debate is still whether the Ca^{2+} that seems to be responsible for LTP induction is of extracellular origin or comes from intracellular Ca^{2+} stores. In a recent study Taube and Schwartzkroin²⁷ have addressed this question using organic Ca^{2+} entry blockers. Their results however, were inconclusive. Izumi et al.¹³, however did show an influence of Ca^{2+} -entry blockers on LTP induction.

The ion channels associated with the *N*-methyl-D-aspartate (NMDA) receptors, a subtype of the glutamate receptors, may gate the entrance of extracellular calcium ions into the cells^{5,19}. The NMDA receptor has been proposed to play a specific role in the initiation process of LTP. It has been shown that NMDA antagonists can specifically block the induction of LTP without affecting the normal field potentials in response to single teststimuli^{4,9}.

A current hypothesis is that NMDA receptor activation induced by tetanization, would open Ca^{2+}

* Present address: Medical Biological Laboratory TNO, P.O. Box 45 2280 AA Rijswijk, The Netherlands.
Correspondence: F.H. Lopes da Silva, Department of General Zoology, Kruislaan 320, Building II, University of Amsterdam, 1098 SM Amsterdam, The Netherlands.

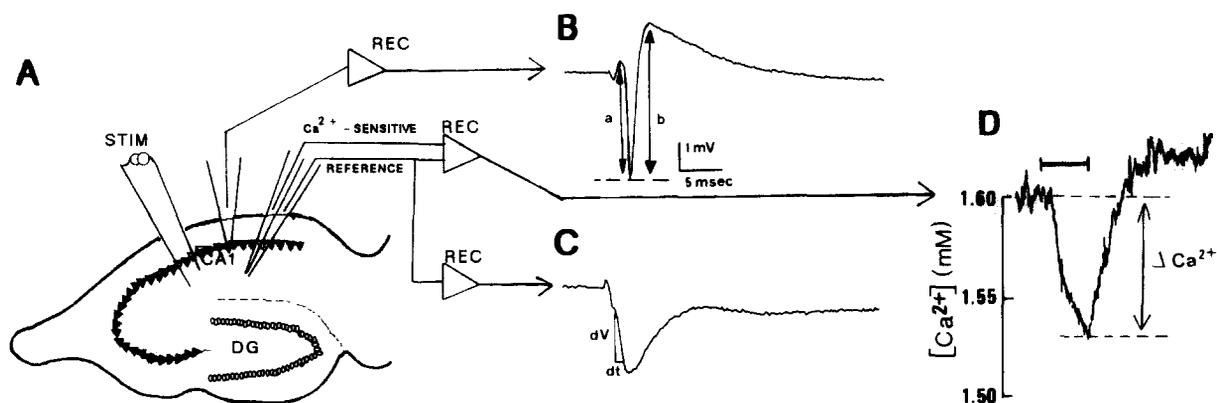


Fig. 1. A: schematic drawing of a hippocampal slice illustrating the positions of the stimulation (STIM) and recording electrodes (REC). B and C: field potentials recorded respectively in the stratum pyramidale (B) and radiatum (C). The value $(a + b)/2$ was taken as the amplitude of the population spike; dV/dt was used to quantify the rising slope of the EPSP. D: an example of a Ca^{2+} signal in response to a tetanus (8 s, 25 Hz) applied to the Schaffer collaterals (indicated by the horizontal bar), recorded in the stratum radiatum by means of the Ca^{2+} -sensitive electrode. The peak of the Ca^{2+} signal (ΔCa^{2+}) was determined as the difference between the peak amplitude and the baseline $[Ca^{2+}]$ as indicated by the broken line.

channels, and in this way be responsible for a large influx of Ca^{2+} into the postsynaptic cells. This would increase the intracellular Ca^{2+} concentration and would be decisive for LTP induction. This hypothesis may imply that the amount of calcium that enters the cells during tetanic stimulation is correlated with the amount of LTP that ensues. In this study we estimated the degree of this correlation. We measured tetanus induced decreases in the extracellular calcium concentration ($[Ca^{2+}]_o$), which are related to the entrance of calcium ions into neurons¹⁰, and thus can be used as a measure of the total amount of calcium that has entered the cells during the tetanus. We have correlated these decreases in $[Ca^{2+}]_o$ with the amount of change in the field potentials which was caused by the tetanic stimulation. In addition, involvement of the NMDA receptor associated channels in this process was studied by investigating the effect of the NMDA antagonist 2-amino-5-phosphonovalerate (2-APV) on both decreases in $[Ca^{2+}]_o$ induced by tetanic stimulation and on LTP.

MATERIALS AND METHODS

Slice preparation and maintenance

Male Wistar rats (160–220 g) were decapitated under ether anaesthesia. The brain was removed and chilled with icecold (0–4 °C) Ringer solution (in mM: NaCl 124, KCl 5, $CaCl_2$ 2, $MgSO_4$ 2, NaH_2PO_4 1.25, $NaHCO_3$ 26 and glucose 10), which was saturated with 95% O_2 –5% CO_2 . The hippocampus was pre-

pared free and transverse slices of about 500 μm were cut by hand with a set of parallel-mounted razorblades. The slices were transferred to a storage chamber in which they were stored at room temperature until used. The storage chamber contained Ringer solution which was constantly gassed with 95% O_2 –5% CO_2 . The slices were allowed at least 1 h of rest before the start of an experiment. At least 15 min before the start of the recordings a number of slices was transferred to the recording chamber. In the recording chamber, which was kept at a temperature of 32–33 °C, the slices were submerged in Ringer solution, saturated with 95% O_2 –5% CO_2 which was perfused at a rate of about 1–2 ml/min.

Stimulation and recording

Stimulation electrodes, consisting of two trimethylated wires of 60 μm diameter were placed in the stratum radiatum of area CA1 in order to stimulate the Schaffer collaterals (Fig. 1). Teststimuli were bipolar, biphasic constant current pulses of 0.2 ms duration and were given every 30 s. The intensity used was always halfway between threshold and the stimulus intensity that elicited a saturated response and ranged between about 50–150 μA . Since Ca^{2+} fluxes, as measured with Ca^{2+} -sensitive microelectrodes, are relatively slow processes, we chose a tetanus duration of 8 s. In order to restrict the total number of pulses we used a tetanus frequency of 25 Hz. Its intensity was equal to the intensity used for the teststimuli.

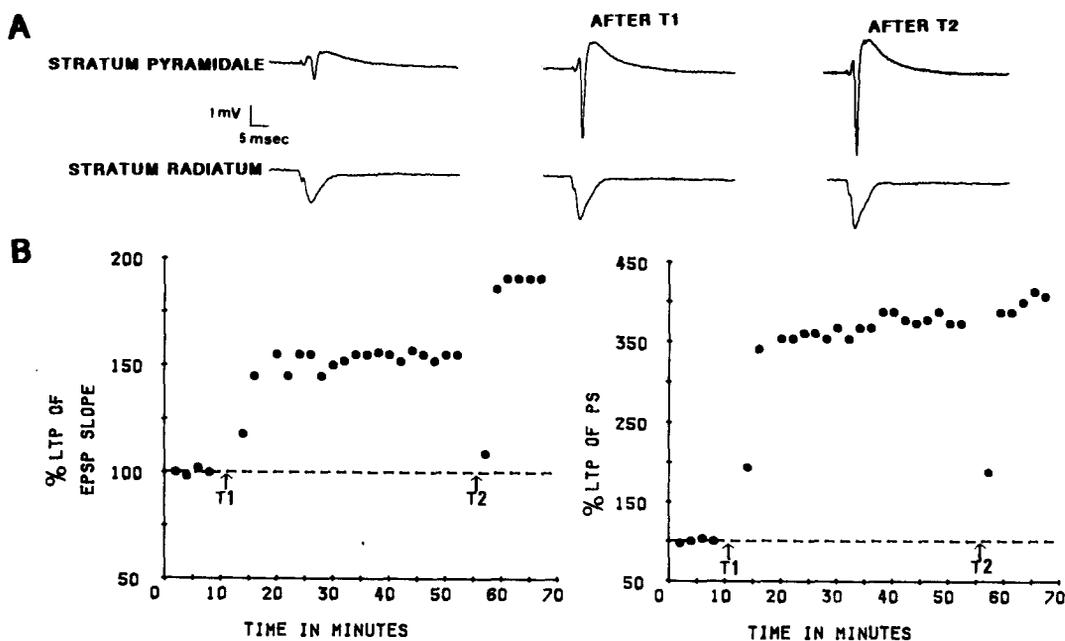


Fig. 2. A: field potentials recorded in the stratum pyramidale (upper row) and the stratum radiatum of a slice from the control group, during the control period, and after the first (T1) and second tetanus (T2). B: plot of the relative amplitude (setting the amplitude during the control period at 100%) of the rising slope of the EPSP (left) and the population spike (PS) (right) of the same slice against time. Arrows indicate the points in time at which the first (T1) and second (T2) tetanus were given. Note that T2 elicited only a small extra increase in field potential.

Field potentials were recorded by means of a glass microelectrode, filled with either 3 M NaCl or 150 mM NaCl (resistance 5–15 M Ω), which was positioned in the stratum pyramidale and by means of the reference barrel of the Ca²⁺-sensitive microelectrode (see below). Since synaptic changes are presumed to be involved in LTP¹, we chose a site within the dendritic tree of the pyramidal cells for this electrode, in the stratum radiatum at about 100 μ m distal to the reversal site of the field excitatory postsynaptic potential (EPSP). It is important to use standard positions of the recording electrodes because of the position dependency of the Ca²⁺ signal²⁹. In order to position the Ca²⁺-sensitive electrode as accurately and reproducibly as possible, the electrode was mounted in a microcomputer controlled stepper motor micromanipulator²³.

Field potentials in response to the teststimuli were sampled at 4 kHz, averaged ($n = 4$) on line using a Motorola Exorset microcomputer and stored on diskette for further analysis.

Calcium-sensitive electrodes

Ca²⁺-sensitive electrodes were made essentially according to the method described by Heinemann et

al.¹¹: electrodes were pulled from theta glass capillaries and the tip was broken to a diameter of 2–5 μ m. The reference barrel was filled with 150 mM NaCl, the ion-sensitive barrel was filled with a 100-mM solution of CaCl₂. The tip of the ion-sensitive barrel was made hydrophobic by means of a 5% solution of trimethyl-chloro-silane in CCl₄, which was sucked into the tip of the electrode. After the tip was cleared of silane, liquid (Ca²⁺) ionexchanger (Fluka) was sucked about 50–100 μ m into the tip.

The Ca²⁺ sensitivity of the electrodes was tested against a number of CaCl₂ solutions with different Ca²⁺ activities in 150 mM of NaCl. Only the electrodes that showed a 25- to 29-mV potential change to a 10-fold change in the [Ca²⁺] were used. The specificity of the Ca²⁺ electrodes for Ca²⁺ over Mg²⁺ and Na⁺ was tested occasionally; they were at least 1000 fold more specific as compared to Mg⁺ and at least 10000 fold as compared to Na⁺.

The electrodes were connected with Ag–AgCl wires to the probe of a high-input impedance amplifier. The differential signal (Ca²⁺-sensitive signal–reference signal) was recorded on a chart recorder. The signal from the reference barrel was used also to record the evoked field potentials from the stratum

radiatum and was therefore fed into the Motorola Exorset microcomputer.

NMDA iontophoresis

A microelectrode with a tip diameter of about $1\ \mu\text{m}$ was filled with a 20-mM solution of NMDA (Sigma) in 150 mM NaCl (pH 7.4). The electrode was glued to a Ca^{2+} -sensitive electrode in such a way that their tips were at a distance of 25–35 μm . The electrode was positioned in the stratum radiatum of area CA1. A retaining current of 10 nA was applied; Ca^{2+} signals were recorded during a iontophoresis pulse which had a duration of 8 s and an amplitude of -75 to -100 nA.

Experimental procedure

The LTP experiments were performed on 29 hippocampal slices. The slices were divided into two groups, the first group ($n = 14$), which we will call hereafter the APV-group, was subjected to the following procedure: after a stable response to the test stimulus was established the perfusion Ringer was changed to a similar Ringer solution with 30 or 50 μM 2-APV (Cambridge Research Biochemicals). After

field potentials were recorded for a period of about 10 min the first tetanus (T1) was applied to the Schaffer collaterals. One min after T1, washout of 2-APV was started. Perfusion with control Ringer was continued for a period of 30–40 min, during which evoked field potentials were recorded every 30 s. After the washout a second tetanus (T2) was applied to the Schaffer collaterals. The recording of evoked field potentials was continued for about 16 min after T2. During both tetani the decrease in $[\text{Ca}^{2+}]_o$ in stratum radiatum was measured with the Ca^{2+} -sensitive electrode.

The second group, the control group ($n = 15$), was subjected to the same experimental procedure except that the perfusion Ringer was not changed and that the time interval between T1 and T2 was usually shorter (15–35 min). In 7 slices of this group field potential recording was continued after T2.

Quantification

The population spike amplitude was determined from the stratum pyramidale responses, as indicated in Fig. 1B. From the stratum radiatum responses (Fig. 1C) the maximal rising slope of the field EPSP

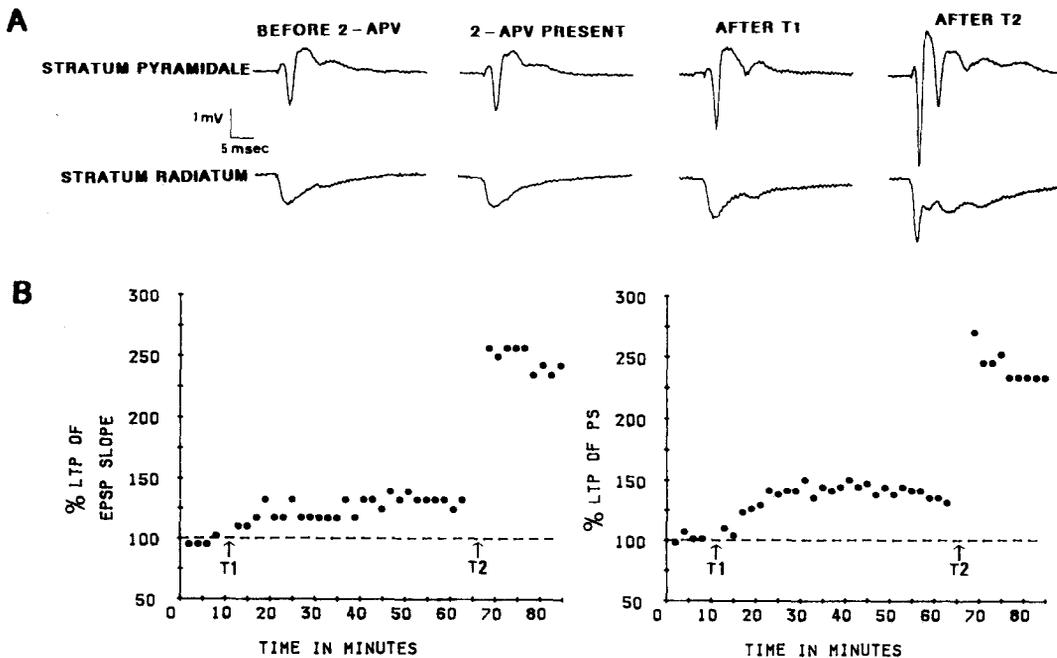


Fig. 3. A: field potentials recorded in the stratum pyramidale (upper row) and radiatum in a slice of the APV group, before and during the period of perfusion with Ringer containing 30 μM 2-APV, and after the first (T1, in the presence of 2-APV) and the second tetanus (T2, after washout of 2-APV) were applied to the Schaffer collaterals. B: plot of the relative amplitude of the rising slope of the EPSP (left) and the population spike (PS) (right), in the same slice, against time. 2-APV was present during the whole control period that is shown, washout was started one minute after T1.

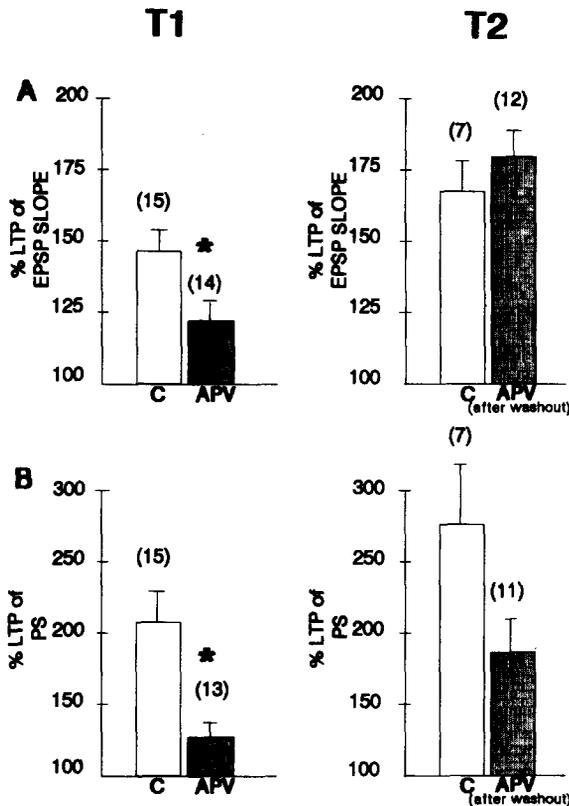


Fig. 4. Average amount of LTP (\pm S.E.M.) after T1 (left) and T2 (right), of the rising slope of the EPSP (A) and the population spike (B) in all slices of the control (C) and the APV group (APV). The response during the control period was set at 100%. APV was only present in the APV group during T1. The asterisk denotes a significant difference ($P < 0.05$, Student's t -test, two-tailed) between the control and the APV group. The total number of slices in each group is indicated between parentheses.

was quantified as indicated. The amount of LTP in the field potentials was determined at 10 min after the tetanus. From the Ca^{2+} signals the peak amplitude was determined as indicated in Fig. 1D.

Statistical testing of the results was done using Student's two-tailed t -test for comparisons between two groups. The Wilcoxon matched-pair signed-rank test was used for comparisons within one group. For the correlation studies the Spearman rank correlation coefficient was computed.

RESULTS

Field potentials

As regards the field potentials our results are in line with earlier reports^{4,9}. In 85% of the slices of the control group T1 elicited LTP of both the population

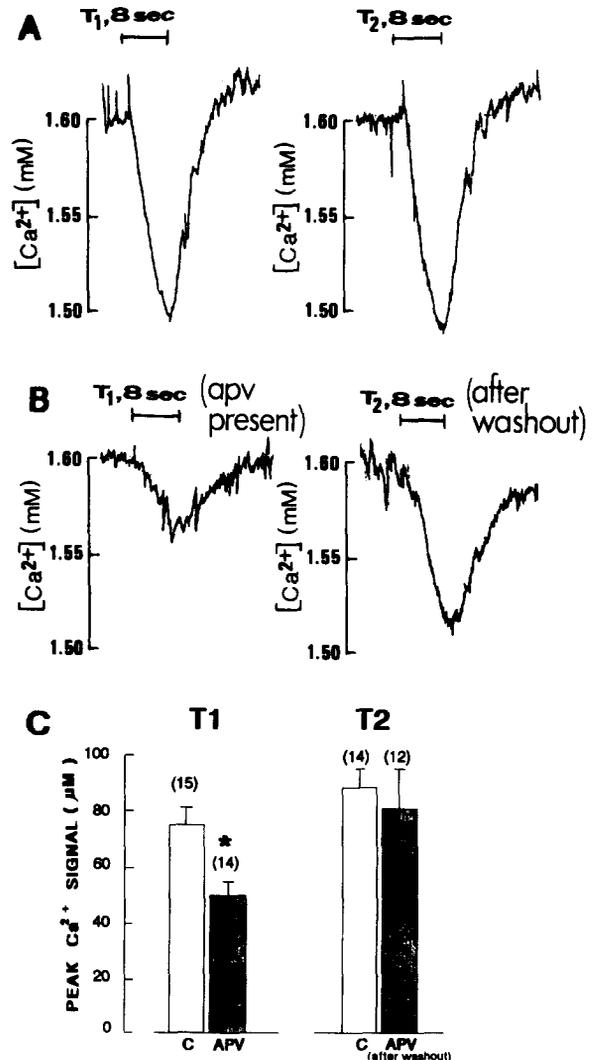


Fig. 5. Examples of decreases in $[\text{Ca}^{2+}]_o$ elicited by T1 (left) and T2 (right) in a slice from the control (A) and from the APV group (B). C. average decrease in $[\text{Ca}^{2+}]_o$ (\pm S.E.M.) for the slices in the control (C) and the APV group (APV), in response to T1 (left) and T2 (right). 2-APV was only present in the slices of the APV group during T1. The number of slices within each group is indicated between parentheses. The asterisk denotes a significant difference between the control and the APV group ($P < 0.02$ Student's t -test, two-tailed). A significant difference was found also between the average decrease in $[\text{Ca}^{2+}]_o$ elicited by T1 and T2 in the APV group ($P < 0.01$, Wilcoxon matched-pairs signed-rank test).

spike (PS) and the field EPSP. A representative example of such an experiment is illustrated in Fig. 2. In general the effect of two successive tetani (T1 and T2) on the EPSP and PS amplitudes is such that after T2 a maximal LTP is approached. The effect of T1 can be quantified relative to the effect of T2. This was done by setting the LTP after T2 to 100%. In 7 slices

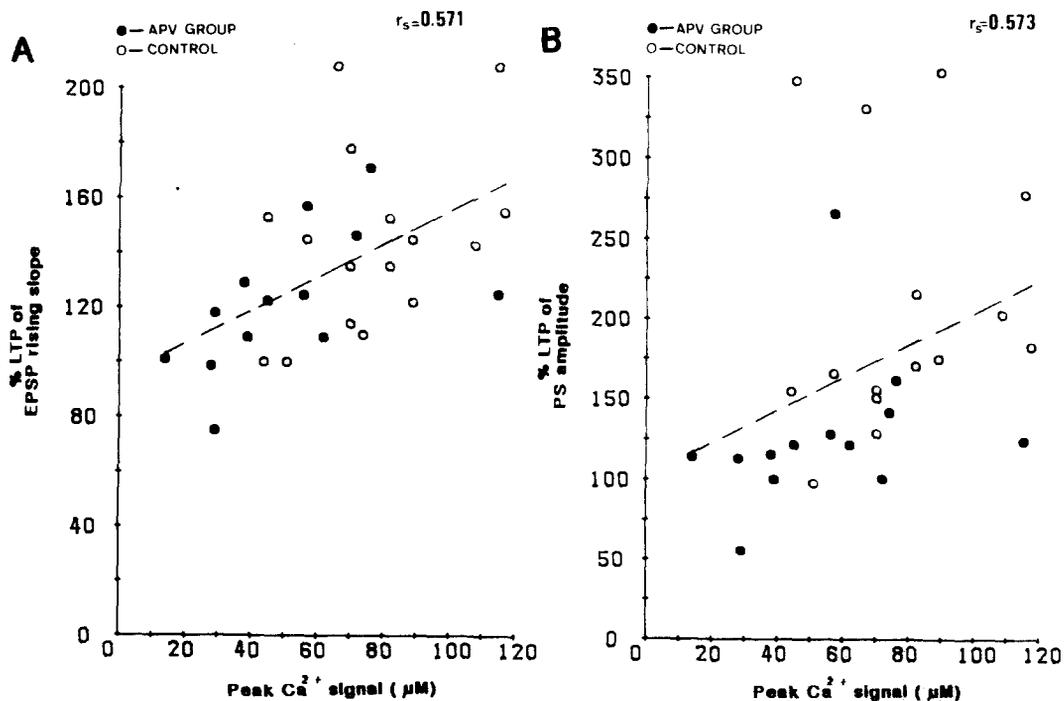


Fig. 6. Correlation between the amplitude of the decrease in $[Ca^{2+}]_o$ and the amount of LTP of the rising slope of the EPSP (A) and of the population spike (B) for all slices. Open circles indicate slices of the control group, filled circles are slices from the APV group. A significant correlation (Spearman rank correlation coefficient $r_s = 0.571$ for the EPSP and $r_s = 0.573$ for the PS, $P < 0.002$) was found between the two variables. The broken line indicates the best linear fit.

of the control group in which the field potentials were monitored also after T2, T1 elicited 78% of the total increase in the field EPSP slope (and 97% for the PS). Thus under these experimental conditions T2 elicited only a slight additional increase in the field potentials.

Fig. 3 shows a similar experiment in which, however, 30 μM of 2-APV was present during T1. 2-APV had no effect on the evoked field potentials during the control period but did reduce the amount of LTP that was elicited by T1: only a small fraction of the total increase in the field potentials after both tetani was induced by T1 (for the slope of the field EPSP 28% and for the PS 38%). Both averages are significantly different from the values obtained in the control group (Student's two-tailed t -test, $P < 0.05$).

The average amount of LTP induced by T1 of both the PS and the slope of the field EPSP was significantly lower in the APV group (Fig. 4). Here we are interested in quantifying the increase induced by T1 in respect to the amplitudes measured during the control period, before T1. Thus we set the amplitude of the EPSP and the PS during this period at 100%, in

contrast with the previous way of quantification. For the EPSP an average value of $121 \pm 7\%$ was found in the APV group whereas this was $146 \pm 8\%$ for the control group ($P < 0.05$, Student's t -test). For the PS the corresponding values were $127 \pm 13\%$ for the APV group and $207 \pm 21\%$ for the controls ($P < 0.05$, Student's t -test). After T2 no significant differences in the amount of LTP were encountered between the two groups.

Stimulus-induced decreases in extracellular calcium concentration

Tetanic stimulation of the Schaffer collaterals resulted in decreases in the $[Ca^{2+}]_o$ from the resting concentration of about 1.6 mM down to about 1.5 mM in slices of the control group (Fig. 5). On average a maximal decrease of $77 \pm 6 \mu\text{M}$ was encountered. This value is in good agreement with the data of Wadman et al.²⁹ recorded in the same preparation at the same position along the cells. The decreases in $[Ca^{2+}]_o$ were, both in the control and in the APV group, often followed by overshoots with respect to the baseline calcium level. In most slices T2 elicited a

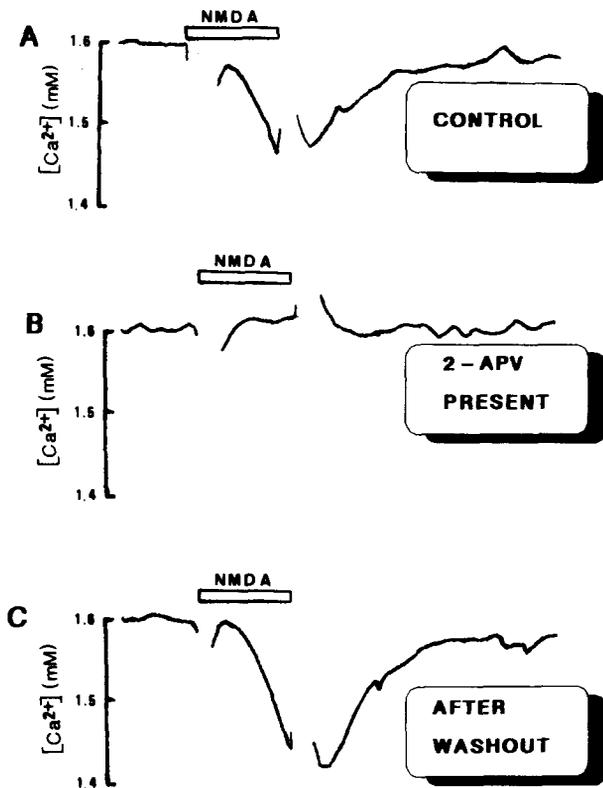


Fig. 7. Decreases in $[Ca^{2+}]_o$ elicited by NMDA iontophoresis (-75 nA, 8 s) before (A), during (B) and after (C) washout of 2-APV (30 μ M). Stimulation artefacts were removed for reasons of clarity.

larger decrease in $[Ca^{2+}]_o$ than T1 (mean decrease: 88 ± 8 μ M) but the difference was generally small and no overall significant difference between the decreases in $[Ca^{2+}]_o$ elicited by T1 and T2 in the control group was encountered.

In Fig. 5B the decreases in $[Ca^{2+}]_o$ induced by T1 and T2 in a slice of the APV group are shown. 2-APV significantly reduced the decrease in $[Ca^{2+}]_o$ induced by T1; a mean value of 52 ± 7 μ M was obtained for the APV-group (Student's *t*-test, $P < 0.02$; Fig. 5C). Furthermore, T2 elicited a much larger decrease in $[Ca^{2+}]_o$ than T1 (average maximal decrease 81 ± 14 μ M; $P < 0.01$, Wilcoxon) in the slices of the APV group. No significant difference was found between the two groups in the decrease of $[Ca^{2+}]_o$ that was induced by T2.

Correlation between tetanus induced decreases in extracellular calcium concentration and LTP

To determine the relationship between the Ca^{2+} signals and LTP, we plotted all changes in $[Ca^{2+}]_o$

and field potentials elicited by T1 from the control and the APV group against each other (Fig. 6). The slices from the APV group cover most of the left part of the scatter plot (small calcium decreases and little LTP) whereas the right part of the plot is taken by the control group. However, in some slices from the 2-APV-treated group an appreciable decrease in $[Ca^{2+}]_o$ could be observed. In such slices a considerable amount of LTP was also induced by T1. Experiments from others^{12,24} suggest that a 30 μ M concentration of 2-APV is sufficient to block all NMDA receptor-mediated processes. We confirmed this by NMDA iontophoresis in 3 additional slices. The decrease in $[Ca^{2+}]_o$ elicited by a NMDA iontophoresis pulse was reversibly blocked by 30 μ M of 2-APV in these slices (Fig. 7).

The decrease in $[Ca^{2+}]_o$ induced by T1 and the corresponding amount of LTP showed a significant positive correlation both with respect to the EPSP slope (Spearman rank correlation coefficient $R_s = 0.571$, $n = 29$, $P < 0.002$) and the PS ($r_s = 0.573$, $n = 28$, $P < 0.002$).

For the slices of the APV-group we calculated the additional amount of LTP as induced by T2 with respect to T1 and the difference between the decreases in $[Ca^{2+}]_o$ that were elicited by T2 and T1. Only the slices of the APV group were used because of the fact that in the control group, T1 elicited a high level of LTP, approaching saturation. A significant positive correlation was found between the increase in the Ca^{2+} signal and the increase in the amount of LTP ($r_s = 0.878$; $n = 12$; $P < 0.001$ for the EPSP and $r_s = 0.802$; $n = 11$; $P < 0.01$ for the PS; Fig. 8).

DISCUSSION

These results confirm the important role of calcium in the processes underlying the initiation of LTP and provide a quantitative correlation between tetanus-induced decreases in $[Ca^{2+}]_o$ and the amount of LTP. This could imply a specific role of calcium in LTP induction. We must note that the correlation coefficients are not large, although significant. This might partly be due to our indirect way of measuring but also indicates that other factors, besides the Ca^{2+} flux during the tetanus, also contribute to the establishment of LTP.

Decreases in $[Ca^{2+}]_o$ presumably reflect the move-

ment of Ca^{2+} ions, along their electrochemical gradient, from the extracellular space into neuronal elements¹⁰. The nature of the overshoots with respect to the baseline calcium level in the Ca^{2+} signals, that were found in many slices, is unclear. These increases in $[\text{Ca}^{2+}]_o$, following the tetanus-induced decreases in $[\text{Ca}^{2+}]_o$, have been observed before^{17,29}; possibly a Ca^{2+} extrusion mechanism is involved¹⁷.

The correlation between the Ca^{2+} influx and LTP suggests that *extracellular* Ca^{2+} is involved in the induction of the phenomenon, which was still an open question²⁷. Although a presynaptic contribution to the Ca^{2+} signal cannot be ignored, the major part of the decrease in $[\text{Ca}^{2+}]_o$ is most likely caused by a flux of Ca^{2+} into postsynaptic elements^{10,14,21,26}. The entrance of a sufficient amount of calcium in the postsynaptic elements and the concomitant rise in the intracellular $[\text{Ca}^{2+}]$ then would lead to the induction of LTP. This gives support to the hypothesis that the induction of LTP is at least in part localized at a postsynaptic site, a process in which the degree of post-

synaptic depolarization seems to be a determining factor^{6,20,30,31}.

The NMDA receptor-associated ionchannels most likely play an important role in LTP induction as is suggested by the action of NMDA antagonists on the phenomenon^{4,9}. During a large depolarization, for instance during a tetanus, the voltage-dependent block by Mg^{2+} ions can be relieved^{12,25} and the channels can become active. The presence of the NMDA receptor antagonist 2-APV reduced the tetanus induced decrease in $[\text{Ca}^{2+}]_o$ by about 40% and also decreased the amount of LTP by a related amount. Therefore it can be concluded that an important part of the tetanus induced decrease in $[\text{Ca}^{2+}]_o$ is mediated by a NMDA receptor-related process. It is likely that the NMDA receptor plays a role in the regulation of the amount of Ca^{2+} that can enter the cell during tetanization. It can be questioned however, whether the NMDA receptor activation plays a decisive role in LTP induction in all cases. In a recent study Harris and Cotman⁸ have shown that LTP can

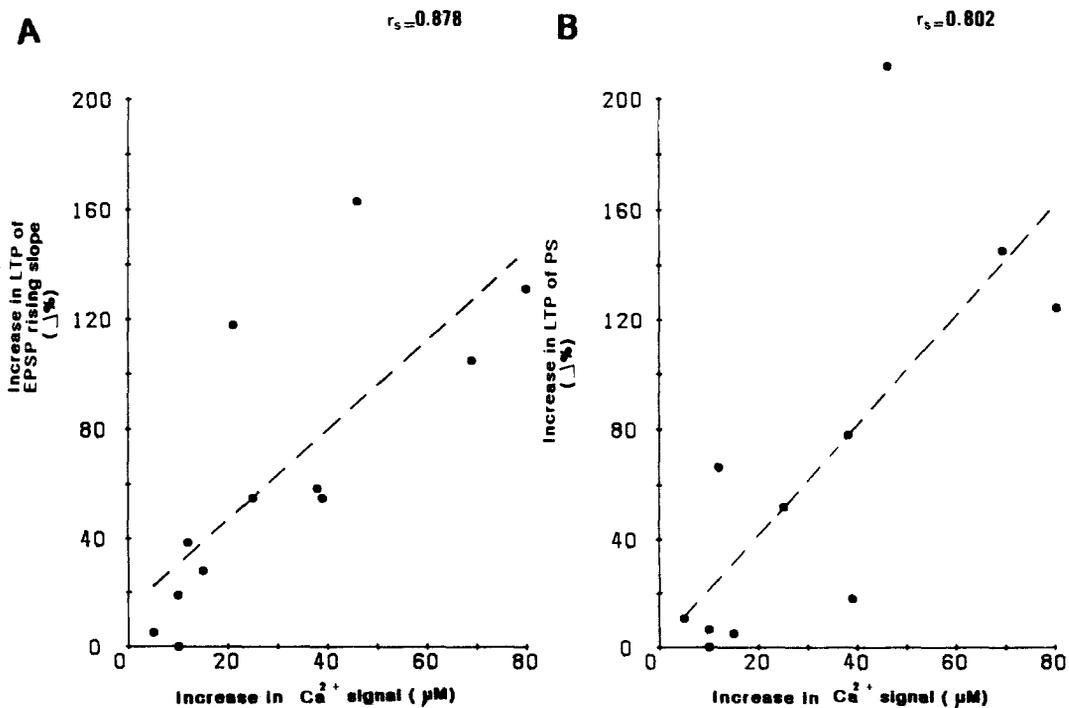


Fig. 8. Correlation between the *increase* in LTP (total amount of change (in % of control value, set at 100%) in field potential after T2 minus the change in field potential encountered after T1) and the *increase* in the Ca^{2+} signal (Ca^{2+} signal elicited by T2 minus the Ca^{2+} signal elicited by T1). Only the data from the slices of the APV group were used. A significant correlation was encountered for both the rising slope of the EPSP (A, $r_s = 0.878$, $P < 0.001$) and for the population spike (B, $r_s = 0.802$, $P < 0.01$). The broken line indicates the best linear fit.

be produced in the mossy fiber-CA3 cell system, in which NMDA receptors are sparse, and that NMDA receptor antagonists have no effect on LTP induction in this system. In our study, in a number of slices of the APV group a tetanus-induced decrease in $[Ca^{2+}]_i$ was found, comparable to that in the slices of the control group, which was accompanied by an appreciable LTP (Fig. 6). Although we cannot rule out the possibility of an incomplete block of the NMDA receptor in these slices, our results suggest that a sufficient Ca^{2+} flux to allow LTP to occur can be reached even if the NMDA receptors are blocked by 2-APV. This may indicate that it is the amount of Ca^{2+} that enters the cell which plays the key role in the devel-

opment of LTP, being under normal circumstances strongly dependent on NMDA receptor activation.

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