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Retrograde signalling with nitric oxide at neocortical synapses

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Abstract

Long-term changes of synaptic transmission in slices of rat visual cortex were induced by intracellular tetanization: bursts of short depolarizing pulses applied through the intracellular electrode without concomitant presynaptic stimulation. Long-term synaptic changes after this purely postsynaptic induction were associated with alterations of release indices, thus providing a case for retrograde signalling at neocortical synapses. Both long-term potentiation and long-term depression were accompanied by presynaptic changes, indicating that retrograde signalling can achieve both up- and down-regulation of transmitter release. The direction and the magnitude of the amplitude changes induced by a prolonged intracellular tetanization depended on the initial properties of the input. The inputs with initially high paired-pulse facilitation (PPF) ratio, indicative of low release probability, were most often potentiated. The inputs with initially low PPF ratio, indicative of high release probability, were usually depressed or did not change. Thus, prolonged postsynaptic activity can lead to normalization of the weights of nonactivated synapses. The dependence of polarity of synaptic modifications on initial PPF disappeared when plastic changes were induced with a shorter intracellular tetanization, or when the NO signalling pathway was interrupted by inhibition of NO synthase activity or by application of NO scavengers. This indicates that the NO-dependent retrograde signalling system has a relatively high activation threshold. Long-term synaptic modifications, induced by a weak postsynaptic challenge or under blockade of NO signalling, were nevertheless associated with presynaptic changes. This suggests the existence of another retrograde signalling system, additional to the high threshold, NO-dependent system. Therefore, our data provide a clear case for retrograde signalling at neocortical synapses and indicate that multiple retrograde signalling systems, part of which are NO-dependent, are involved.

Introduction

Communication between nerve cells is classically considered a process which is restricted to the synapses and occurs in one direction, from the pre- to the postsynaptic cell (Eccles, 1964). Whilst this holds true for the main information flow, several lines of evidence suggest a possibility for fast intercell signalling mediated by diffusible molecules (Garthwaite, 1991; Snyder, 1992; Garthwaite & Boulton, 1995; Hölscher, 1997). This kind of signalling is not constrained to the synaptic structures and a message emitted by one cell can be received by any other neuron which is located close enough and has appropriate receptors. Retrograde signalling is a necessary mechanism for bridging the gap between postsynaptic induction and the partially presynaptic maintenance of long-term potentiation (LTP) in the hippocampus (Bliss & Collingridge, 1993; Malenka & Nicoll, 1993). Several molecules have been suggested as candidates for diffusible messengers, including arachidonic acid (Williams *et al*., 1989), nitric oxide (Gally *et al*., 1990; Böhme *et al*., 1991; O'Dell *et al*., 1991; Schuman & Madison, 1991), carbon monoxide (Zhuo *et al*., 1993) and platelet-activating factor (Kato *et al*., 1994). Among these molecules, the nitric oxide has received most attention (Garthwaite & Boulton, 1995; Hölscher, 1997). Apart from acting as a retrograde messenger in some forms of hippocampal LTP, NO may also play a role in long term depression (LTD) or in modulation of synaptic plasticity (Izumi *et al*., 1992; Izumi & Zorumski, 1993; Kato & Zorumski, 1993). Signalling with NO is also exploited in the cerebellum, where it is involved in LTD of synaptic transmission between parallel fibres and Purkinje cells (Shibuki & Okada, 1991; Daniel *et al*., 1993; Lev-Ram *et al*., 1995, 1997; Boxall & Garthwaite, 1996; Blond *et al*., 1997). In that case NO is produced in the climbing fibres or in interneurons, and acts postsynaptically on the Purkinje cells. Recent studies suggest involvement of NO-signalling pathway in LTD in the striatum (Calabresi *et al*., 1999), and in LTP in some areas of the neocortex (Nowicky & Bindman, 1993; Wakatsuki *et al*., 1998; Haul *et al*., 1999). One common feature of NO-mediated signalling is that it can be prevented by bath application of NO scavengers (e.g. O'Dell *et al*., 1991; Schuman & Madison, 1991; Shibuki & Okada, 1991). Thus, the signalling is intercellular, and the messenger molecule diffuses from one cell to the other. This suggests the possibility that the messenger molecule could also affect those synapses which did not participate in the activity that led to the messenger production and release. Indeed, LTP induction is often accompanied by heterosynaptic changes, which occur locally (Bonhoeffer *et al*., 1989; Kossel *et al*., 1990; Schuman & Madison, 1994; Engert & Bonhoeffer, 1997, 1999) and at larger distances (Lynch *et al*., 1977; Tsumoto, 1992; Scanziani *et al*., 1996) from the tetanized synapses. In fact, most of the plasticity-inducing protocols might activate only a subset of the several thousands of synapses innervating any cortical cell, while the majority of synapses

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might remain silent. This makes investigation of mechanisms of heterosynaptic plasticity a necessary step towards understanding the behaviour of plastic neuronal networks. It has been demonstrated that postsynaptic challenges, like intracellular tetanization (Kuhnt *et al*., 1994; Volgushev *et al*., 1994, 1997, 1999) or light-induced release of caged calcium inside the cell (Neveu & Zucker, 1996) can induce long-term changes in synaptic transmission, which occlude tetanus- or pairing-induced LTP. Some recent data suggest that these forms of synaptic plasticity are associated with presynaptic changes (Volgushev *et al*., 1997) thus implying retrograde signalling. In the present study we investigated properties of retrograde signalling at neocortical synapses, in particular its dependence on the strength of the postsynaptic challenge and on the interruption of the NO signalling pathway.

Some of the results have been presented in abstract form (Volgushev *et al*., 1998).

Materials and methods

Wistar rats (4–6-week-old) were anaesthetized with ether, decapitated, and slices of the visual cortex were prepared in an ice-cold solution. The perfusion medium contained (in mM): 125 NaCl, 2.5 KCl, 1.5 $MgSO_4$, 2 $CaCl_2$, 1.25 Na_2HPO_4, 25 $NaHCO_3$ and 25 D-glucose (pH 7.4) bubbled with 95% O_2 and 5% CO_2. Intracellular recordings from layer 2/3 pyramidal cells were obtained with sharp microelectrodes (2.5 M potassium acetate or 1.2 M potassium acetate with 1% biocytin) under submerged conditions at 30 °C. After amplification (Axoclamp 2A) the data were fed into a computer (PC-486; Digidata-1200; PClamp software: Axon Instruments, Foster City CA, USA). Figure 1 shows a scheme of the locations of stimulation and recording electrodes within the slice and a general outline of the experimental protocol. Two inputs to a cell were activated in alternation, at 0.05–0.08 Hz. Both inputs were tested with paired-pulse stimuli, applied with a 50-ms interpulse interval. Control responses were recorded for 20–40 min, then synaptic stimulation was stopped and intracellular tetanization was applied. After that, the responses were recorded for another 0.5–2 h. Cells which were lost < 30 min after the tetanus were not included in further analysis. Intracellular tetanization consisted of one or three trains (1/min) of 10 bursts (1/s) of 20 depolarizing pulses (0.5–1.8 nA, 10 ms, 50 Hz). The current was adjusted so that the first 3–7 pulses in a burst evoked spikes (Fig. 1). No synaptic stimuli were applied during the intracellular tetanization, which was therefore a purely postsynaptic challenge. Input resistance was tested either with small hyperpolarizing pulses (0.1–0.2 nA, 10 ms), or was estimated from current–voltage relationships. Only cells with stable input resistance and resting membrane potential were subjected for the further analysis. It has been demonstrated that intracellular tetanization (Kuhnt *et al*., 1994; Volgushev *et al*., 1994, 1997), as well as photolytic rise of postsynaptic [Ca^{2+}] (Neveu & Zucker, 1996), can induce long-term changes in synaptic transmission, which occlude tetanus-induced and pairing-induced plasticity (Kuhnt *et al*., 1994; Neveu & Zucker, 1996; Volgushev *et al*., 1999). EPSP amplitudes, paired pulse facilitation (PPF) ratio and coefficient of variation (*CV*) were measured off-line (Volgushev *et al*., 1997). The EPSP amplitudes were measured as the difference between the mean membrane potential within two windows of 1–3 ms width, one positioned on the rising slope, before the peak of the averaged EPSP or over the peak, and another one immediately before the response. LTP and

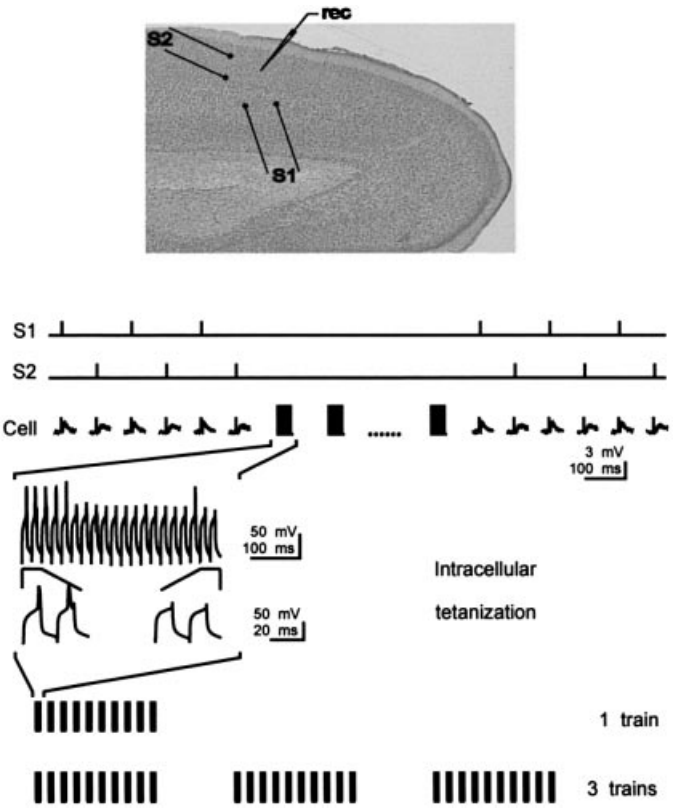

FIG. 1. Experimental protocol. Inset at top shows positioning of the stimulation (S1 and S2) and recording electrodes in a slice of the rat visual cortex. Test stimuli were applied in alteration at two stimulation sites. Intracellular tetanization consisted of trains of bursts of short depolarizing pulses. A typical example of the membrane potential trace during application of one burst of depolarizing pulses is shown in the lower inset.

LTD were estimated as changes of the amplitude of the first response in a paired-pulse paradigm. As a control, data from the 10–15-min period preceding the tetanization were used. Only those cells in which the response amplitude remained stable during that period were used for further analysis. A few cases (< 15% of all recorded inputs) of responses which showed clear trends or extreme amplitude variability throughout the recording period were also discarded. For plotting time courses of the response amplitude changes the responses were normalized to the averaged control values. For plotting summary graphs the normalized responses were averaged across experiments. For correlation analysis the magnitude of post-tetanic changes was assessed from responses averaged over the 10-min period where amplitude changes were maximal (potentiation or depression), starting 10–50 min after tetanization (Markram & Tsodyks, 1996). For the inputs which did not express statistically significant amplitude changes, data were taken from the latest available 10-min interval. Paired-pulse facilitation ratio was estimated as a ratio between the mean amplitude of the second and of the first responses during the respective periods. For calculation of the coefficient of variation (*CV*) the responses to the first stimulus in each pair of paired stimuli were used. The reversed coefficient of variation was calculated as CV^{-2} = (mean amplitude)^2/(Variance)

Variance was corrected for the noise:

Variance = (Response variance) – (Variance of the noise).

Statistical analysis was based on Student's *t*-test and Wilcoxon–Mann–Whitney test.

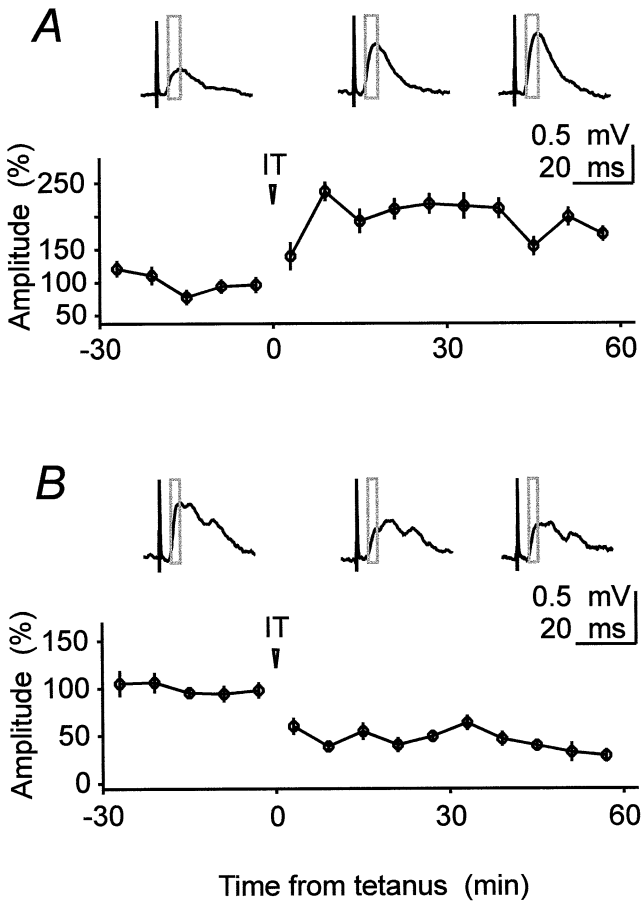

FIG. 2. Example of long-term potentiation (A) and long-term depression (B) induced by 3 trains of intracellular tetanization in a visual cortical cell. Time course of the amplitude changes of averaged responses ($n = 25$). Application of intracellular tetanization (IT) is marked with arrowhead. Insets show averaged postsynaptic potentials ($n = 50$) evoked before, 10–25 min and 30–45 min after intracellular tetanization, with a superimposed window for the response amplitude measurement. A and B show responses of the same cell evoked by alternating electric stimulation applied through S1 (A) and S2 (B) electrodes (see Fig. 1). In this and the following figures error bars are shown when larger than symbol size.

Results

We studied changes in synaptic transmission to layer 2–3 pyramidal cells in slices of the rat visual cortex evoked by intracellular tetanization: trains of bursts of short suprathreshold depolarizing pulses applied to the cell through the recording microelectrode (Kuhnt *et al*., 1994; Volgushev *et al*., 1994, 1997) (Fig. 1). The results presented in this study are based on the analysis of 215 inputs to 130 cells. These cells had stable membrane potentials below –70 mV and stable input resistance throughout the experiment, and the synaptic responses did not change significantly during the control period. Synaptic responses had amplitudes of 0.5–2 mV, and therefore were produced by stimulation of a few presynaptic fibres. Spikes were never observed during testing stimulation, indicating that autapses did not contribute to the test responses. In 85 cells both inputs fulfilled stability criteria and were used for the analysis, and in 45 cells one input was analysed. We performed three main series of experiments. We studied the effects of (i) a prolonged, three-train intracellular tetanization; (ii) a short, one-train intracellular tetanization; and (iii) a prolonged tetanization during blockade of NO signalling pathway.

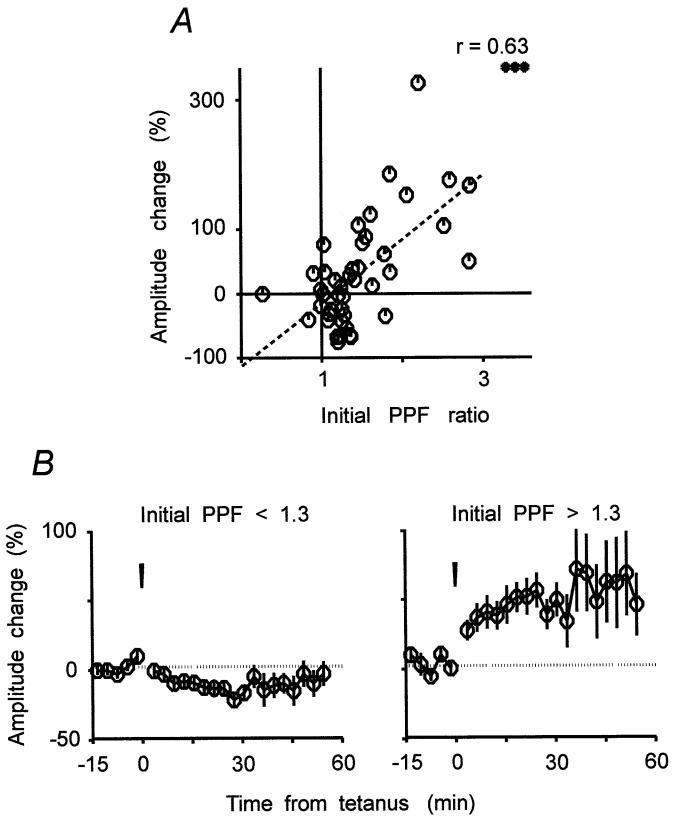

FIG. 3. Long-term changes in synaptic transmission induced by 3 trains of intracellular tetanization depend on initial state of presynaptic release mechanisms. (A) Correlation between post-tetanic amplitude change and initial paired-pulse facilitation (PPF) ratio ($n = 43$). (B) Time course of mean amplitude changes in inputs with initially low (< 1.3) and initially high (> 1.3) PPF ratio. In this and the following figures: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$. r, coefficient of correlation.

Effects of a prolonged intracellular tetanization on synaptic transmission

Three trains of intracellular tetanization led to a significant increase (Fig. 2A) or decrease (Fig. 2B) of the amplitude of excitatory postsynaptic potentials (EPSPs) in 32 of 43 inputs. These changes in response amplitude were robust, long-term (Fig. 2) and were not associated with any appreciable change of the passive membrane properties, as indicated by the stability of the membrane potential and the input resistance before and after tetanization (data not shown).

We observed both potentiation and depression after intracellular tetanization similar to results obtained with photolysis of caged Ca^{2+} compounds (Neveu & Zucker, 1996). The direction and the magnitude of the amplitude change was related to the initial PPF ratio: inputs with initially high PPF had a tendency to be potentiated, whilst inputs with initially low PPF were most often depressed or did not change. This dependence was documented by three sets of observations. First, there was a significant correlation between the initial PPF ratio and the amplitude change for all inputs (Fig. 3A, $n = 43$). Second, when the inputs were segregated into two subgroups of nearly equal size ($n = 21$ and $n = 22$) according to the initial PPF ratio, the inputs with initial PPF > 1.3 were, on average, potentiated, whilst the inputs with initial PPF < 1.3 were, on average, depressed (Fig. 3B). We stress here that this segregation of the inputs into two groups was made exclusively on the basis of their initial PPF ratio, with no respect to the effect of intracellular tetanization. Finally, initial PPF ratio was significantly higher ($P < 0.01$) in the group of

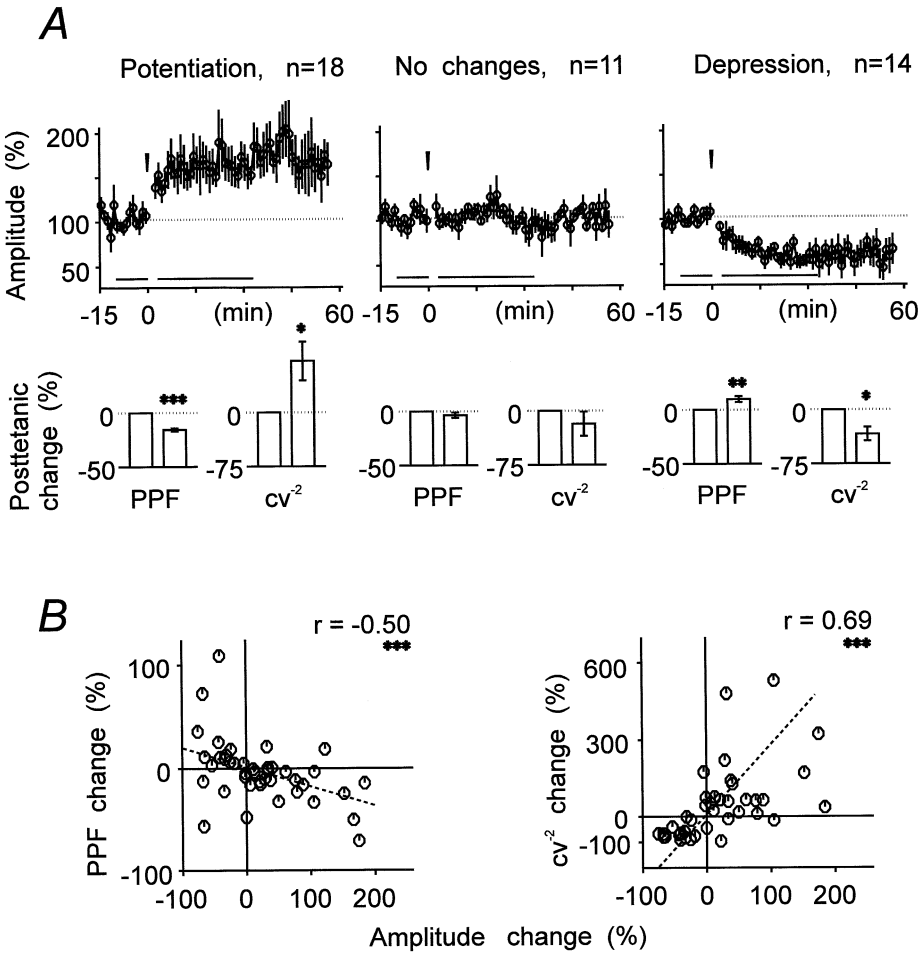

FIG. 4. Long-term changes in synaptic transmission induced by 3 trains of intracellular tetanization involve presynaptic changes. (A) Time course of mean response amplitude changes and averaged changes of PPF ratio and CV^{-2} for the periods indicated in the time course for the inputs which underwent potentiation, depression or did not change after intracellular tetanization. Note that both potentiation and depression are associated with significant changes of PPF ratio and reversed coefficient of variation. (B) Correlation of the post-tetanic change in PPF ratio and CV^{-2} with the amplitude change for all inputs tested with 3 trains of intracellular tetanization in control conditions (n = 43). Note significant correlation between changes of both, PPF and reversed coefficient of variation on the one hand, and response amplitude changes on the other.

potentiated inputs than in the group of the inputs which were depressed after intracellular tetanization.

In those cases where two inputs to the same cell were recorded simultaneously, the amplitude changes induced by intracellular tetanization in the two inputs were not correlated (coefficient of correlation, r = 0.05, n = 18 pairs, $P > 0.1$). Furthermore, the same tetanization could lead to potentiation of one input and depression of another input to the same cell (n = 6, example in Fig. 2). Therefore, long-term changes induced by intracellular tetanization depended on the properties of a particular input but not on some general properties of the postsynaptic cell or on peculiarities of reaction of individual cells to the intracellular tetanization.

Three trains of intracellular tetanization led to a LTP in 18 inputs and to LTD in 14 out of 43 inputs studied (Fig. 4A). The remaining 11 inputs did not change significantly after intracellular tetanization. The potentiation of the response amplitude induced by three trains of intracellular tetanization was associated with a significant decrease of the PPF ratio to 84 ± 1.4% of the pretetanic value and a significant increase of the reversed coefficient of variation (CV^{-2}) to 171 ± 27.1% (Fig. 4A). Depression was associated with an opposite change of these two parameters: an increase of PPF ratio to 111 ± 2.7% and a decrease of CV^{-2} to 66 ± 9.5% of the control (Fig. 4A). Furthermore, for the whole sample (n = 43) there was a significant negative correlation between changes of PPF and the amplitude change (r = –0.50, n = 43, $P < 0.001$) and a significant positive correlation between changes of CV^{-2} and the amplitude change (r = 0.69, $P < 0.001$) (Fig. 4B). PPF ratio and CV^{-2} are two independent parameters which are believed to reflect the state of presynaptic release mechanisms (Katz & Miledi, 1968; Zucker, 1989; Faber & Korn, 1991; Regehr *et al*., 1994; Schulz, 1997). Both these parameters changed significantly and in close correlation with the induction of potentiation and depression, strongly suggesting that presynaptic mechanisms are involved in the maintenance of long-term changes in synaptic transmission, potentiation being associated with an increase and depression with a decrease of release probability (Katz & Miledi, 1968; Zucker, 1989; Faber & Korn, 1991; Voronin, 1993; Kullman, 1994; Regehr *et al*., 1994; Stevens & Wang, 1994; Schulz, 1997).

It has recently been suggested (Wang & Kelly, 1996) that changing the desensitization level of postsynaptic glutamate receptors could lead to changes of the PPF ratio, and thus imitate presynaptic changes. To test for that possibility we reduced AMPA receptor desensitization with aniracetam. Bath application of aniracetam led to an increase of the amplitude of the EPSPs in a dose-dependent manner (Fig. 5). However, this increase of the response amplitude was not accompanied by any alteration of the PPF. In 15 experiments with aniracetam application the response amplitude increased to 116 ± 1.6% of the control ($P < 0.001$), but PPF ratio remained unchanged (99 ± 2.4% of the control, $P > 0.1$). It should be noted here that the response amplitude was measured around the EPSP peak, as in the plasticity experiments. Later EPSP components were affected more strongly by aniracetam application, but this was not relevant in the context of our present study. Thus, alterations of

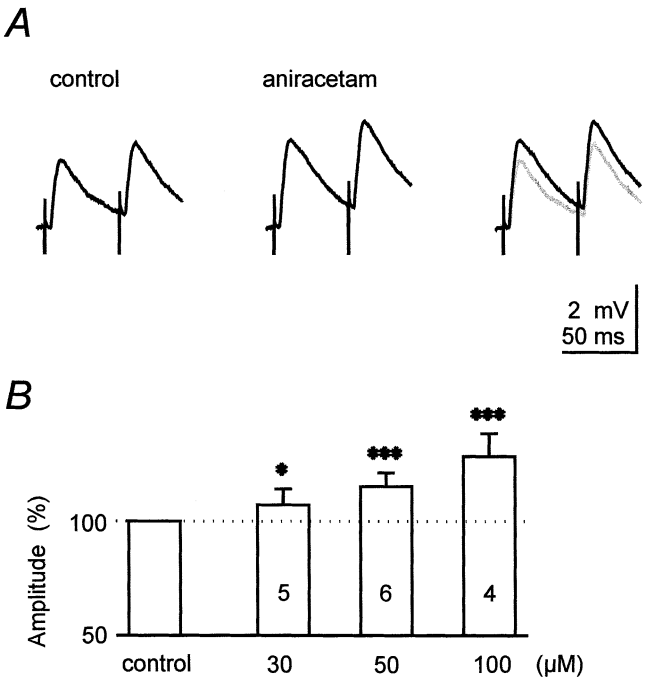

FIG. 5. Aniracetam, which reduces desensitization of AMPA-receptors, increases the response amplitude in a dose-dependent way. (A) Averaged responses (n = 50) evoked by paired-pulse stimuli in control solution and in a solution containing 100 μM aniracetam. On the right panel responses before and after aniracetam application are superimposed. (B) Dependence of the increase of response amplitude on concentration of aniracetam. Number of experiments with each concentration is indicated within the bars.

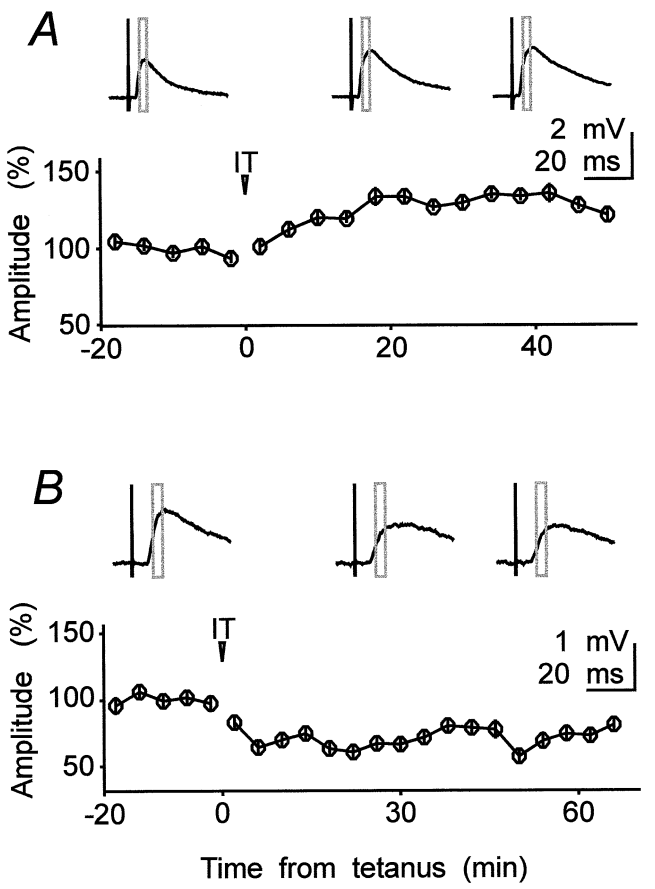

FIG. 6. Example of long-term potentiation (A) and depression (B) induced by 1 train of intracellular tetanization. Time course of the amplitude changes of averaged responses (n = 20), and averaged postsynaptic potentials (n = 50) evoked before, 10–25 min and 30–45 min after intracellular tetanization. Other conventions as in Fig. 2. Data in A and B are from two different cells.

the desensitization level of AMPA receptors could not account for the changes of the PPF ratio in our experimental situation.

Therefore, although postsynaptic changes may have contributed to the response amplitude changes, they cannot explain the observed changes in PPF and CV^{-2}. Because the presynaptic changes occurred after a purely postsynaptic challenge, they must have resulted from retrograde signalling. The retrograde signal activated by three trains of intracellular tetanization (i) could induce presynaptic changes which supported robust modifications of EPSP amplitude (ii) could influence presynaptic release in both directions, and (iii) induced presynaptic changes with the polarity and the magnitude depending on the initial state of release mechanisms.

Effects of a short intracellular tetanization on synaptic transmission

One train of intracellular tetanization of similar intensity led to a significant increase (Fig. 6A) or a decrease (Fig. 6B) of the amplitude of test EPSPs in 28 out of 41 inputs. This potentiation (n = 17) and depression (n = 11) shared a number of common features with the synaptic changes induced by a prolonged tetanization. Both potentiation and depression induced by a single train of intracellular tetanization were long-lasting (Fig. 6). When two inputs to the same cell were recorded, no significant correlation was found between amplitude changes in the two inputs (r = 0.09, n = 15 pairs, $P > 0.1$). Furthermore, the LTP and LTD induced by one train of intracellular tetanization involved some presynaptic changes. This is indicated by a significant decrease of the PPF ratio (90 ± 2.9%) associated with potentiation, a significant increase of CV^{-2}, the reversed coefficient of variation, after potentiation (162 ± 14.9%) and a significant decrease of CV^{-2} associated with depression (72 ± 9.8%) (Fig. 7A). Moreover, for all inputs tested with one train of intracellular tetanization (n = 41), there was a significant correlation between the response amplitude changes on the one hand and the PPF change (r = –0.50, $P < 0.001$) or CV^{-2} changes (r = 0.46, $P < 0.001$) on the other (Fig. 7B).

However, long-term changes of synaptic transmission induced by a single train of intracellular tetanization were different in several important aspects from the LTP and LTD induced by a prolonged tetanization. First, both potentiation and depression were of a significantly ($P < 0.01$) smaller amplitude than those induced by three trains (compare black and grey symbols in Fig. 7A). Second, the direction and the magnitude of the amplitude changes induced by a single train of intracellular tetanization did not depend on the initial PPF ratio (Fig. 8). There was no correlation between the initial PPF ratio and the amplitude change (Fig. 8A). There was no significant difference of the effect of a single train of intracellular tetanization onto two subgroups of inputs, segregated according to the initial PPF ratio (PPF > 1.3 or PPF < 1.3, Fig. 8B). Furthermore, the groups of the potentiated and of the depressed inputs did not differ in their initial PPF ratio ($P > 0.1$).

This difference between the effects of one and of three trains of intracellular tetanization was not due to sample differences in initial PPF ratio or in the magnitude of the amplitude changes. The correlation between the initial PPF and the amplitude change after

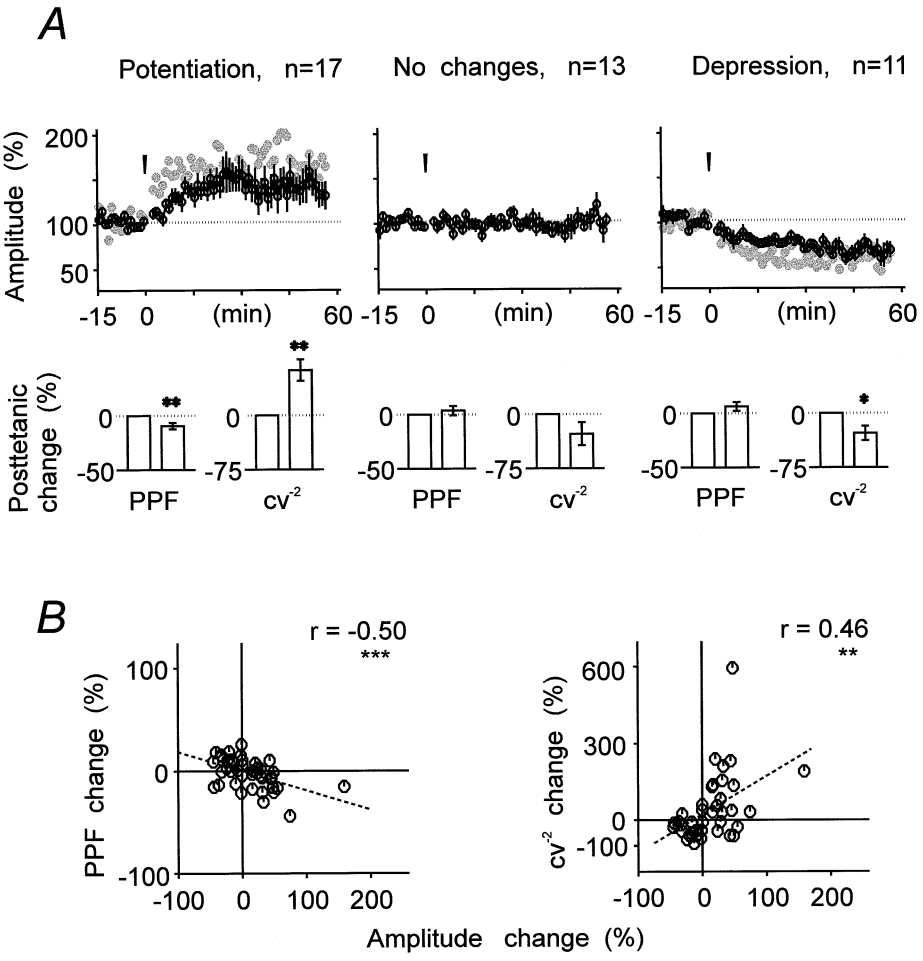

FIG. 7. Long-term changes in synaptic transmission induced by 1 train of intracellular tetanization involve presynaptic changes. (A) Time course of mean response amplitude changes and averaged changes of PPF ratio and CV^{-2} for the periods of maximal amplitude changes for the inputs which underwent potentiation, depression or did not change after intracellular tetanization. For comparison, time course of potentiation and depression induced by 3 trains of intracellular tetanization (data from Fig. 4A) is shown as grey symbols, without s.e.m. bars. (B) Correlation of the post-tetanic change in PPF ratio and CV^{-2} with the amplitude change for all inputs tested with 1 train of intracellular tetanization ($n = 41$). Note significant correlation between changes of both, PPF and reversed coefficient of variation on the one hand, and response amplitude changes on the other.

three trains of intracellular tetanization remained significant ($P < 0.05$) for subpopulations of inputs with a moderate initial PPF (< 2.0) or with moderate amplitude changes (–50 to +75%).

To test whether the changes of synaptic transmission observed after a single train of intracellular tetanization were really induced by the tetanization or whether they occurred just by chance we made the following analyses. For each cell, the mean response amplitude was estimated during three equally long and equally spaced intervals. The duration of these intervals was 7–15 min depending on the length of recording of the control responses in a particular cell. Two intervals were positioned before the tetanus, the second one just preceding the tetanization. The third interval was positioned immediately after the tetanization. Then we compared the amplitude changes which occurred between the first and the second interval (no tetanization in between, 99.1 ± 7.8%, $n = 41$) to those which took place between the second and the third interval, separated by the tetanization (100.9 ± 19.9%, $n = 41$). The variance of the amplitude changes was significantly higher ($P < 0.005$) in the latter case, indicating that one train of intracellular tetanization led to the response amplitude changes, which cannot be accounted for by chance. To check for the possibility that the higher variance of the response amplitude changes after one train of intracellular tetanization as compared to the control period was not due to our initial selection of cells for the analysis (see Materials and methods), we repeated the above test for all cells in which one train of intracellular tetanization was applied. The complete sample of 60 inputs included 12 inputs which were recorded for < 30 min after the tetanization and seven inputs which did not fulfil stability criteria. Even for that complete sample, the variance of the response amplitude changes which occurred before the tetanization was significantly lower than the variance of changes which took place across the tetanization (97.2 ± 13.2% vs. 99.4 ± 21.7%, $P < 0.01$, $n = 60$). Although this does not completely exclude the possibility of a contribution of chance-guided variability, the above results strongly suggest that the response amplitude changes observed after a single train of intracellular tetanization were indeed related to the tetanus.

Thus, a single train of intracellular tetanization was capable of inducing LTP or LTD. Because these changes of synaptic transmission involved some presynaptic mechanisms, a retrograde signalling cascade must have been activated. This retrograde signalling differed from the signalling activated by the prolonged tetanization in the following respects: (i) it had a lower activation threshold; (ii) it supported presynaptic changes of a smaller magnitude; and (iii) its effect was not related to the initial state of presynaptic release mechanisms.

Interruption of the NO-signalling pathway alters the effects of the prolonged intracellular tetanization on synaptic transmission

To evaluate possible biochemical difference between the two retrograde signals, we repeated the experiments while interrupting the NO-signalling pathway, by blocking the NO-synthase activity with the inhibitors N(G)-Nitro-L-arginine methyl ester (L-NAME) or N(ω)-Nitro-L-arginine (NOArg), or by preventing the extracellular

TABLE 1. Influence of the blockade of NO-signalling pathway on the characteristics of synaptic changes induced by intracellular tetanization

	3 IT trains	1 IT train	3 IT trains + L-NAME	3 IT trains + NOArg	3 IT trains + PTIO	3 IT trains + Hb	3 IT trains + L-NAME or NOArg	3 IT trains + PTIO or Hb
LTP (% of control)	192 ± 16.5	142 ± 7.2	141 ± 7.0	154 ± 9.1	165 ± 16.4	128 ± 2.9	147 ± 5.6	151 ± 11.5
LTD (% of control)	52 ± 4.7	67 ± 2.9	59 ± 7.9	59 ± 3.4	66 ± 5.7	53 ± 4.9	59 ± 4.9	58 ± 4.0
Correlation between amplitude change and								
Initial PPF	0.63***	0.04	0.00	–0.36	0.28	–0.33	–0.13	–0.02
PPF change	–0.50***	–0.50***	–0.45**	–0.34	–0.60***	–0.55**	–0.39**	–0.52***
CV^{-2} change	0.69***	0.46**	0.50**	0.70***	0.39*	0.63***	0.45***	0.44**
Number of analysed inputs	43	41	33	26	28	25	(59)	(53)

IT, intracellular tetanization; L-NAME, N(G)-Nitro-L-Arginine Methyl Ester; NOArg, N(ω)-Nitro-L-Arginine; PTIO, 2-(4-Carboxyphenil)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide; Hb, Haemoglobin. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; two-tail correlation tests.

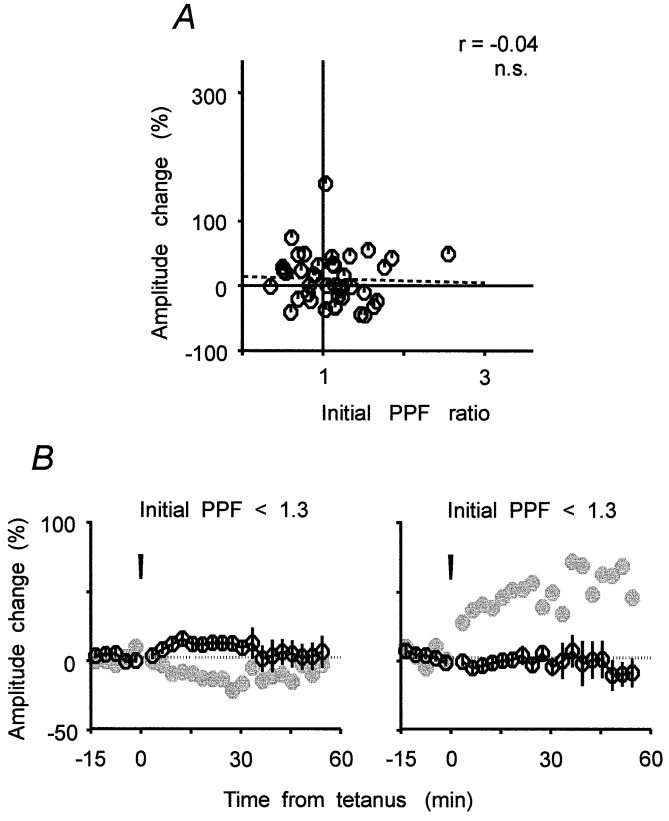

FIG. 8. Long-term changes in synaptic transmission induced by 1 train of intracellular tetanization did not depend on initial state of presynaptic release mechanisms. (A) Relation between post-tetanic amplitude change and initial PPF ratio ($n = 41$). (B) Time course of mean amplitude changes in inputs with initially low (< 1.3) and initially high (> 1.3) PPF ratio. For comparison, time course of the amplitude changes induced by 3 trains of intracellular tetanization (data from Fig. 3B) is shown as grey symbols, without s.e.m. bars.

spread of nitric oxide with the NO-scavengers 2-(4-Carboxyphenil)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) or haemoglobin (Hb).

Bath application of L-NAME or carboxy-PTIO differently affected basal synaptic transmission. Application of 20 μM L-NAME led to a small but significant decrease of the amplitude of synaptic responses, to 89 ± 1.6% of the control level ($n = 11$, $P < 0.001$). In contrast, application of 20 μM carboxy-PTIO led to a significant increase of the amplitude of the test EPSPs, to 111 ± 1.7% of the control ($n = 14$, $P < 0.001$). In both cases, the response amplitude changes were not accompanied by any significant changes of the PPF, which was 101 ± 2.2% and 100 ± 2.2% of the control. Therefore, although bath application of L-NAME or carboxy-PTIO can lead to small changes of the EPSP amplitude, these changes of the basal synaptic transmission were, most probably, not associated with significant modifications of the state of the release mechanisms.

In the next series of experiments we studied effects of three trains of intracellular tetanization on synaptic transmission under the conditions of blockade of the NO signalling pathway. Experiments were performed with two inhibitors of NO synthase activity, L-NAME (20 μM, $n = 33$) and NOArg (20 μM, $n = 26$) and two NO scavengers, carboxy-PTIO (20 μM, $n = 28$) and haemoglobin (5 μM, $n = 25$). In the presence of any of these substances in the recording medium, both long-term potentiation and long-term depression could be induced by intracellular tetanization. Neither the frequency of occurence of potentiation and depression, nor their magnitude, differed significantly in these four series of experiments. Intracellular tetanization led to potentiation of synaptic transmission in 11 out of 33 experiments with L-NAME, 10 out of 26 experiments with NOArg, 10 out of 28 experiments with carboxy-PTIO and in six out of 25 experiments with haemoglobin. The response amplitude increased on average to 141 ± 7.0%, 154 ± 9.1%, 165 ± 16.4% and 128 ± 2.9% of the control, respectively. Depression was observed in seven experiments with L-NAME, seven experiments with NOArg, seven experiments with carboxy-PTIO and in nine experiments with haemoglobin. The amplitude of EPSPs decreased to 59 ± 7.9%, 59 ± 3.4%, 66 ± 5.7% and 53 ± 4.9% of control, respectively. Two further observations point to the similarity of the long-term changes of synaptic transmission induced by intracellular tetanization under conditions of the interruption of the NO signalling pathway with L-NAME, NOArg, carboxy-PTIO or haemoglobin. First, presynaptic changes are likely to accompany modifications of the response amplitude induced in the presence of any of these compounds, as indicated by the correlations between the amplitude changes on the one hand, and changes of the PPF ratio or of the CV^{-2} on the other. These correlations were significant in all but one case (Table 1). Second,

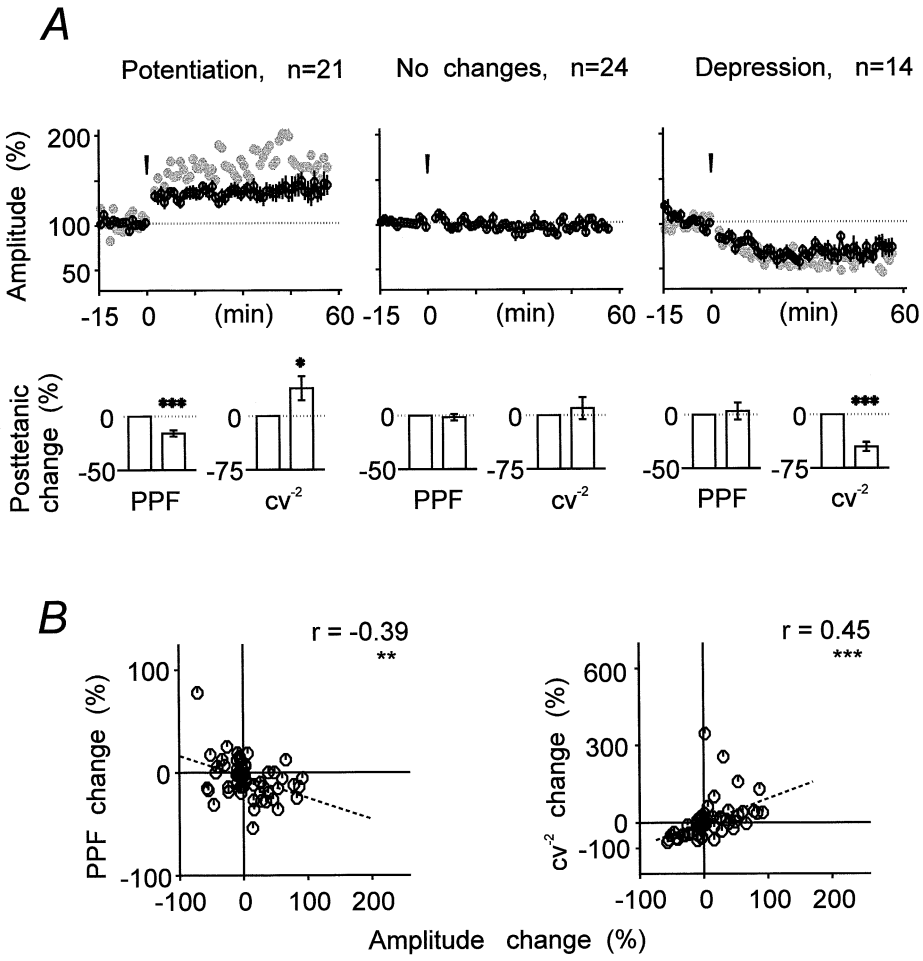

FIG. 9. Long-term changes in synaptic transmission induced by 3 trains of intracellular tetanization during blockade of NO-synthase activity involve presynaptic changes. (A) Time course of mean response amplitude changes and averaged changes of PPF ratio and CV^{-2} for the periods of maximal amplitude changes for the inputs which underwent potentiation, depression or did not change after intracellular tetanization. NO synthase activity was blocked by bath application of 20 (μM L-NAME or 20 (μM NOArg. For comparison, time course of potentiation and depression induced by 3 trains of intracellular tetanization under control conditions (data from Fig. 4A) is shown as grey symbols, without s.e.m. bars. (B) Correlation of the post-tetanic change in PPF ratio and CV^{-2} with the amplitude change for all inputs tested with 3 trains of intracellular tetanization during blockade of NO synthase activity with L-NAME or NOArg ($n = 59$). Note significant correlation between changes of both, PPF and reversed coefficient of variation on the one hand, and response amplitude changes on the other.

with any of the four tested substances in the bath solution the direction and the magnitude of the amplitude changes did not correlate with initial PPF ratio. Common features allowed us to pool the results obtained with the two NO-synthase inhibitors in one group and the results obtained with the two NO scavengers in the second group.

Long-term changes of synaptic transmission, induced by intracellular tetanization under conditions of interruption of the NO-signalling cascade with NO-synthase inhibitors or NO scavengers, were associated with significant changes of the PPF ratio and reversed coefficient of variation. Long-term potentiation induced in the presence of NO-synthase inhibitors in the bath (Fig. 9A) was accompanied by a significant decrease of the PPF ratio to 84 ± 2.9% of the control value ($P < 0.001$, $n = 21$) and a significant increase of the CV^{-2} to 139 ± 16.8% of the control ($P < 0.05$). During the long-term depression, the PPF ratio had a tendency to increase (103 ± 7.9%, $n = 14$), whilst the CV^{-2} significantly decreased to 54 ± 6.5% of the control ($n = 14$, $P < 0.001$). In the presence of NO scavengers (Fig. 10A) long-term increase of the response amplitude was associated with significant decrease of the PPF ratio to 77 ± 3.8% of the control value ($P < 0.001$, $n = 16$), and a significant increase of the CV^{-2} to 170 ± 12.1% of the control ($P < 0.01$). The depression was associated with the opposite changes of the two parameters. The PPF ratio increased to 119 ± 8.8% ($P < 0.05$, $n = 16$) and the CV^{-2} decreased to 70 ± 10.3% of the control ($P < 0.01$, $n = 16$).

The PPF changes were significantly inversely correlated with the changes of the amplitude of synaptic responses ($r = –0.39$, $P < 0.01$, $n = 59$ for the group with NO-synthase inhibitors and $r = –0.52$, $P < 0.001$, $n = 53$ for the group with NO scavengers). Changes of the reversed coefficient of variation were significantly positively correlated with the amplitude changes in the two groups ($r = 0.45$, $P < 0.001$ and $r = 0.44$, $P < 0.01$).

These data strongly suggest that long-term potentiation and depression induced by intracellular tetanization under conditions of blockade of the NO-signalling pathway were associated with respective increase and decrease of the release probability.

Under conditions of blockade of the NO signalling pathway the response amplitude changes were not related to the initial state of release mechanisms. There was no significant correlation between the amplitude change and the initial PPF ratio in any of the four experimental groups or in the pooled data for NO synthase inhibitors or NO scavengers (Table 1). No significant differences were found between the effects of intracellular tetanization on the inputs with initially strong or initially weak PPF, or between the initial PPF ratios of potentiated and depressed inputs.

Therefore, the retrograde signal activated by the prolonged intracellular tetanization under conditions of blockade of the NO-signalling pathway (i) was able to influence presynaptic release bidirectionally, and thus to support both long-term potentiation and long-term depression, but (ii) its effect was not related to the initial state of the presynaptic release mechanisms.

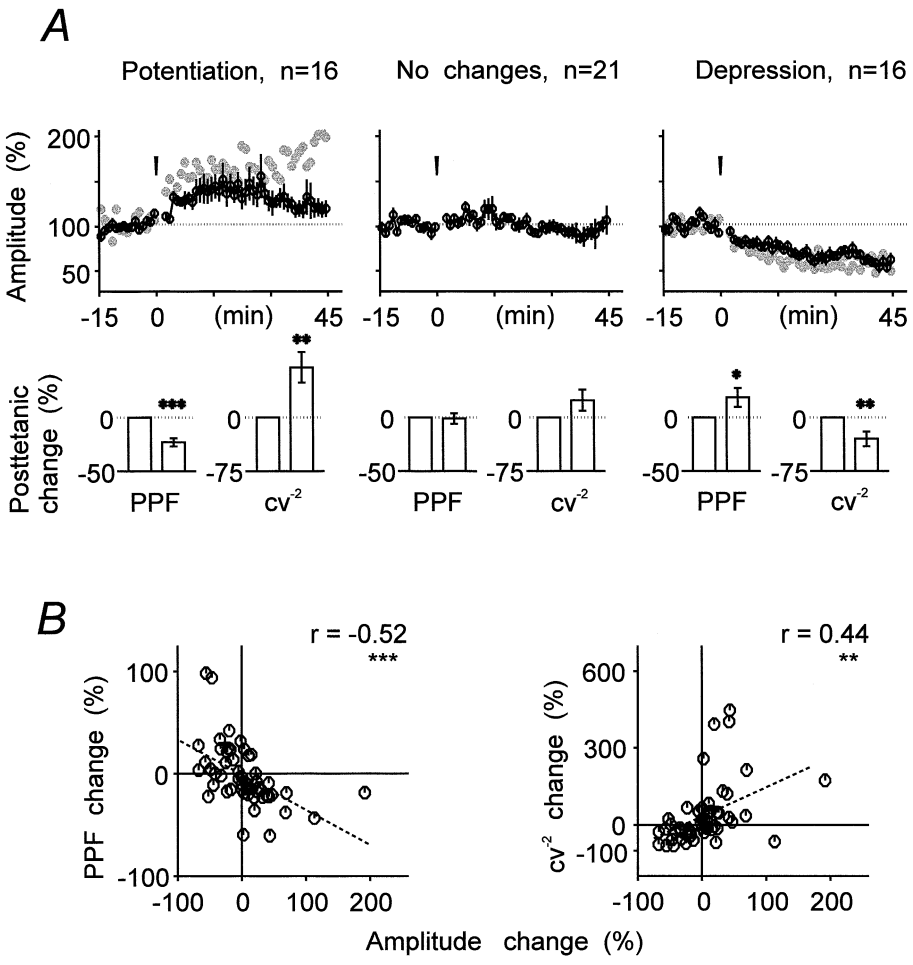

FIG. 10. Long-term changes in synaptic transmission induced by 3 trains of intracellular tetanization during bath application of NO scavengers involve presynaptic changes. (A) Time course of mean response amplitude changes and averaged changes of PPF ratio and CV^{-2} for the periods of maximal amplitude changes for the inputs which underwent potentiation, depression or did not change after intracellular tetanization. NO synthase activity was blocked by bath application of 20 (μM carboxi-PTIO or 5 (μM Haemoglobin. For comparison, time course of potentiation and depression induced by 3 trains of intracellular tetanization under control conditions (data from Fig. 4A) is shown as grey symbols, without s.e.m. bars. (B) Correlation of the post-tetanic change in PPF ratio and CV^{-2} with the amplitude change for all inputs tested with 3 trains of intracellular tetanization during blockade of NO signalling pathway by bath application of NO scavengers carboxy-PTIO or Hb (n = 53). Note significant correlation between changes of both, PPF and reversed coefficient of variation on the one hand, and response amplitude changes on the other.

Discussion

The present results demonstrate that: (i) presynaptic changes can be induced by a purely postsynaptic challenge; (ii) retrograde signalling can support presynaptic changes in either direction, i.e. potentiation or depression; (iii) multiple retrograde signalling systems with different properties operate at neocortical synapses; and (iv) part of the retrograde signalling at neocortical synapses depends on the nitric oxide-mediated pathway.

A purely postsynaptic challenge can induce presynaptic changes in either directions

Several observations indicate that long-term changes of synaptic transmission induced by intracellular tetanization were associated with changes in release. First, induction of LTP or LTD was associated with significant changes of two independent indices of presynaptic release, PPF ratio and reversed coefficient of variation. Changes in both these indices are indicative of an increased release probability at potentiated synapses, and a decrease of the release probabilty at depressed synapses (Katz & Miledi, 1968; Zucker, 1989; Faber & Korn, 1991; Voronin, 1993; Kullman, 1994; Regehr *et al*., 1994; Stevens & Wang, 1994; Schulz, 1997). Second, the direction and the magnitude of changes of the PPF ratio and CV^{-2} were significantly correlated with the direction and the magnitude of the response amplitude changes. This indicates a close relationship between changes in release and long-term changes of the response amplitude. Third, changes in one further correlate of release, the number of response failures, have been reported to accompany induction of LTP or LTD by intracellular tetanization (Volgushev *et al*., 1997). Finally, results of control experiments with aniracetam demonstrated that changing the level of desensitization of AMPA receptors did not affect PPF ratio. Hence, changes of indices of presynaptic release could not have been mimicked by alteration of desensitization of postsynaptic receptors in our experimental situation. Altogether, these observations strongly suggest that LTP and LTD induced by intracellular tetanization were associated with presynaptic modifications.

The intracellular tetanization can be considered a purely postsynaptic challenge, because synaptic connections made by a single layer 2/3 pyramidal cell onto other cells are too weak to lead to spike generation (e.g. Thomson & West, 1993), and because autapses are unlikely to contribute to our test responses. Occurrence of presynaptic changes after a purely postsynaptic challenge implies involvement of retrograde signalling. Notably, our results indicate that presynaptic changes and thus retrograde signalling accompanied induction of both long-term potentiation and long-term depression in the rat visual cortex.

Data from the hippocampus indicate that maintenance of LTP of synaptic transmission to the CA1 pyramidal cells has a presynaptic component (Bekkers & Stevens, 1990; Malinow & Tsien, 1990; Kullmann & Nicoll, 1992; Schulz *et al*., 1994; Stevens & Wang, 1994; Malgaroli *et al*., 1995; but see Manabe & Nicoll, 1994). Because the LTP is induced postsynaptically, the presynaptic changes might result from activation of the retrograde signalling (Gally *et al*.,

1990; Williams *et al*., 1993a). There is also evidence for a decrease of the release probability associated with induction of LTD (Bolshakov & Siegelbaum, 1994; Stevens & Wang, 1994; Xiao *et al*., 1994; Goda & Stevens, 1996). Our results demonstrate that at neocortical synapses retrograde signalling can accompany induction of both LTP and LTD. Thus, neocortical cells are endowed with mechanisms which allow a postsynaptic cell to achieve both up- and down-regulation of transmitter release at synapses a cell receives. An important feature of the retrograde signalling at neocortical synapses is that the retrograde signal is not only necessary, but also sufficient, for induction of presynaptic changes. Activation of a presynaptic fibre is not a necessary step for retrograde signalling and presynaptic changes to occur. This greatly expands the possibilities for heterosynaptic plasticity, making not only the synapses that are located within the reach of a diffusible retrograde signalling molecule released from the active synapses, but essentially all synapses at a cell a potential target for presynaptic changes. As a consequence, the input specificity of the plastic synaptic changes becomes a function of the activation duration and/or strength. The synaptic changes induced by a short and weak postsynaptic activation, like the pairing procedure or weak afferent tetanization, would then remain local, with the rule of input specificity broken only at short distances (Bonhoeffer *et al*., 1989; Kossel *et al*., 1990; Schuman & Madison, 1994; Engert & Bonhoeffer, 1997). Prolonged and strong postsynaptic activation should lead to massive heterosynaptic changes. This conjecture is supported by the data showing that both in the CA1 area of the hippocampus (Grover & Teyler, 1990) and in the neocortex (Aroniadou & Teyler, 1992; Aroniadou *et al*., 1993) a very strong and prolonged tetanization induces a form of LTP which is independent of NMDA receptor activation, but is blocked by antagonists of voltage-dependent calcium channels. Further, also in the hippocampus, long-term synaptic changes can be induced by purely postsynaptic challenges, like raising intracellular [Ca^{2+}] (Neveu & Zucker, 1996) or intracellular tetanization (Kuhnt *et al*., 1994).

Multiple retrograde signalling systems, some of which depend on a NO-mediated pathway, operate at neocortical synapses

Presynaptic changes accompanied the long-term changes of synaptic transmission induced in all of the experimental situations that we have studied: by strong or weak intracellular tetanization, and under conditions of interruption of the NO-signalling pathway. Hence, the retrograde signalling occurred in all these cases. However, retrograde signalling, activated in the different experimental situations had markedly different properties, suggesting that multiple retrograde signalling systems operate at neocortical synapses.

A qualitative difference between the effect of the prolonged (stronger) intracellular tetanization and the short (weaker) tetanization was observed, in the dependence of the direction and the magnitude of response amplitude changes on the initial transmitter release properties. The direction of the amplitude change correlated to the initial PPF ratio, when induced by the prolonged but not by the short tetanization. Because this difference cannot be accounted for by various degrees of activation of the same retrograde signalling system, it implies that short and prolonged intracellular tetanization activated two different systems. A system whose effect on the presynapse depended on the initial state of the release mechanisms, operated only after the stronger (prolonged) tetanization, and thus might have a higher activation threshold. The high threshold system appeared to be NO-dependent. Its operation required both normal functioning of NO synthase and undisturbed spreading of NO between the cells, because either inhibition of NO synthase activity or bath application of NO scavengers eliminated the dependence of the effect of the strong intracellular tetanization on the initial state of release mechanisms. These considerations allow us to suggest that at neocortical synapses NO is exploited as a messenger molecule by the high threshold retrograde signalling system, which modifies the presynapse depending on its initial state.

A possibility that NO could mediate the opposite effects on synaptic transmission is supported by several lines of evidence. NO can modulate transmitter release in either direction (Garthwaite & Boulton, 1995). It has been demonstrated that, in the hippocampus, NO can increase the frequency of spontaneous events (O'Dell *et al*., 1991) and potentiate (Arancio *et al*., 1996) or suppress (Boulton *et al*., 1994) the EPSP amplitude. In the cerebellum NO decreases EPSPs evoked in Purkinje cells by parallel-fibre stimulation (Shibuki & Okada, 1991; Daniel *et al*., 1993; Blond *et al*., 1997), although here it acts postsynaptically. Our data show that bath application of chemical substances which affect the NO signalling can either increase or decrease the synaptic responses in the neocortex. Interestingly, whilst inhibition of the NO synthase activity and prevention of NO spread led to modifications of the basal synaptic transmission in the opposite directions, both groups of compounds had a similar effect on the outcome of the intracellular tetanization. This may suggest different concentrations of NO and/or different targets for NO action during the basal transmission as compared to the intracellular tetanization. In the former case, NO at low concentration may act predominantly within the producing cell, whilst during the tetanization higher NO concentrations might be reached, leading to a diffusion across the cell membrane and to the spread to the neighbouring neurons. At hippocampal synapses, the effect of NO depends on the pattern of synaptic activity, and thus NO can play a dual role in regulation of synaptic transmission (Zhuo *et al*., 1993, 1994). Our results indicate that this conjecture may hold for neocortical synapses too. Furthermore, at neocortical synapses retrograde signalling which presumably involves the NO-pathway can induce presynaptic changes even without synaptic activity. The direction and the magnitude of the induced synaptic changes depended on the actual state of the release mechanisms, which is, in turn, a function of previous synaptic activity. Operation of a retrograde signalling system with such properties is well suited to account for 'metaplasticity' (Abraham & Bear, 1996), that is for dependence of an effect of a plasticity-inducing challenge on the history of synaptic activation. One further functional consequence of the operation of a system with such properties would be a normalization of release probability over nonactive synapses. Indeed, in those inputs where release probability was low (high PPF), it tends to increase, and in the inputs where release probability was high (low PPF) it tends to decrease. This could preclude both a runaway potentiation of the strong synapses and a rapid elimination of the weak connections. Rather, this procedure would keep the release probabilities in the middle range, preserving susceptibility for modifications in either direction. Notably, normalization procedure is a necessary attribute of any model of learning networks.

A source of NO in neocortical plasticity could be one of the isoforms of NO synthase. Direct measurements revealed NO production by cortical cells in association with synaptic stimulation (Wakatsuki *et al*., 1998) or application of NMDA (Kojima *et al*., 1998). Neuronal NO synthase has been localized in the neocortex, although the density of NOS-positive cells is very low (Rodrigo *et al*., 1994; Sugaya & McKinney, 1994; Estrada & DeFelipe, 1998; Wakatsuki *et al*., 1998). Evidence from the hippocampus indicates that endothelial rather than neuronal NO synthase is responsible for the NO production during induction of LTP (O'Dell *et al*., 1994; Kantor *et al*., 1996; Son *et al*., 1996; Wilson *et al*., 1999). Prominent

immunoreactivity for endothelial NO synthase was found in the CA1 pyramidal cells (Dinerman *et al*., 1994). The recent finding that induction of LTP in the neocortex is impaired in mice deficient in endothelial NO synthase (Haul *et al*., 1999), indicates that this isoform could be responsible for the bulk of NO production in neocortical neurons. In our experimental situation NO production might take place in pyramidal cortical cells, because during the intracellular tetanization protocol the activation was, with all likelihood, restricted to a single postsynaptic cell. Which isoform of NO synthase is responsible for this production remains to be identified.

Presynaptic changes occurred even when the NO-dependent pathway was interrupted, or after short tetanization, indicating that retrograde signalling could involve pathways additional to the NO–cGMP system. Together with the data demonstrating the existence of a NO-synthesis-independent form of LTP in the hippocampus (Gribkoff & Lum-Ragan, 1992; Haley *et al*., 1993), this helps to explain the controversial results of experiments in which involvement of NO in retrograde signalling during LTP induction was studied (Williams *et al*., 1989; O'Dell *et al*., 1991; Schuman & Madison, 1991; Gribkoff & Lum-Ragan, 1992; Haley *et al*., 1992; Izumi *et al*., 1992; Williams *et al*., 1993a, 1993b; Bannerman *et al*., 1994). In addition to segregation between the NO-dependent and NO-independent pathways of retrograde signalling, there could be a further dichotomy, for example between systems supporting potentiation and depression. Evidence for operation of several retrograde signalling systems at neocortical synapses is supplementary to the results which suggest existence of multiple mechanisms of synaptic plasticity (Malenka & Nicoll, 1993; Teyler *et al*., 1994; Hawkins, 1996). These data, together with recent reports on retrograde signalling at inhibitory synapses (Alger & Pitler, 1995) and plasticity of inhibitory synaptic transmission (Komatsu, 1994; Marty & Llano, 1995), show that both excitatory and inhibitory synapses in the cerebral cortex have a repertoire of mechanisms for bidirectional communication between the pre- and postsynaptic neuron allowing for a precise coordination of activity of the multiple biochemical cascades which control the synaptic gain.

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Abbreviations

AMPA, α–amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; carboxy-PTIO, 2-(4-Carboxyphenil)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; *CV*, coefficient of variation; EPSP, excitatory postsynaptic potential; Hb, haemoglobin; L-NAME, N(G)-Nitro-L-arginine methyl ester; LTD, long-term depression; LTP, long-term potentiation; NMDA, N-Methyl-D-aspartic acid; NO, nitric oxide; NOArg, N(ω)-Nitro-L-arginine; PPF, paired-pulse facilitation; *r*, coefficient of correlation.

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