Neuroscience Letters, 81 (1987) 291–295
Elsevier Scientific Publishers Ireland Ltd.

NSL 04891

Long-lasting potentiation produced by a phorbol ester in the hippocampus of the anaesthetized rat is not associated with a persistent enhanced release of excitatory amino acids

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(Received 4 March 1987; Revised version received 9 June 1987; Accepted 4 July 1987)

Key words: Long-term potentiation (LTP); Push–pull; High-pressure liquid chromatography (HPLC); Glutamate; Aspartate; Phorbol ester; Hippocampus; Rat

The relationship between the long-lasting enhancement of synaptic transmission produced by a phorbol ester and the release of endogenous excitatory amino acids has been investigated in the CA_1 hippocampal region of the anaesthetized rat. Using the push–pull technique, the concentration of glutamate and aspartate was assayed in the perfusate by high-pressure liquid chromatography. Application of phorbol 12 13 diacetate produced a long lasting enhancement of the field excitatory postsynaptic potential (EPSP) (over 2 h). This was associated with a brief (10 min) significant increase in the release of glutamate and aspartate. However, subsequently the levels of the amino acids in the perfusate were not different from the pre-drug (control) levels although the field EPSP was still enhanced. It is concluded that the long-lasting enhancement produced by phorbol ester is not due to a persistent increase in the release of excitatory amino acids.

Protein kinase C (PKC) is a phosphorylating enzyme which plays an important role in a variety of biological functions, notably in the expression of neuronal plasticity in the vertebrate [10] and invertebrate [5] nervous system. Recent studies have shown that in the hippocampal slice preparation, application of phorbol esters, which are known to activate PKC [3], produces a long-lasting enhancement of synaptic transmission [6]. The effect of phorbol esters is not associated with changes in postsynaptic cell excitability, suggesting that the long-lasting increase of the excitatory postsynaptic potential (EPSP) may result from a sustained enhancement of transmitter release, i.e. that phorbol esters act presynaptically. This and other observations suggest that this long-lasting enhancement is indistinguishable from the clas-

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sical long-term potentiation (LTP) which is observed after a train of high-frequency electrical stimulation [1] and considered to be a useful model of memory [11].

In the present study, using a push–pull device, we have examined the relationship between the long-lasting enhancement of synaptic transmission produced by a phorbol ester and the release of endogenous glutamate and aspartate.

Twelve adult Wistar rats weighing 200–300 g were used for the experiments. They were anaesthetized with urethane (2 g/kg) and placed in a stereotaxic frame. A push–pull cannula was introduced in one hippocampus (A = 4; L = 2.8). To ascertain the exact position of the cannulae we relied on the typical field potential produced by the electrical stimulation of the commissural pathway. For this purpose, a recording monopolar electrode (50 μm o.d) which has the same length as the inner cannula was attached to the outer cannula (0.5 mm o.d). A twisted bipolar electrode was also implanted in the hippocampal commissure to stimulate the commissural pathway (0.05 ms duration, 0.1 Hz). Once the push–pull cannula was accurately placed, artificial cerebrospinal fluid was perfused at a flow rate of 10 μl/min; with the following composition (in mM): 126.5 NaCl; 2.4 KCl; 2 $CaCl_2$; 0.83 $MgCl_2$; 0.5 KH_2PO_4; 0.5 Na_2SO_4; 27.5 $NaHCO_3$; 5.9 glucose, pH 7.4. The perfusion was continued for a stabilization period of 1 h. At the end of this period, samples were collected at 5-min intervals during 1 h (control period) and a pulse of phorbol 12–13 diacetate was applied for 5 min; samples were collected at 5-min intervals for an additional period of 1 or 2 h. The content of glutamate and aspartate in samples were determined by HPLC, using a procedure similar to that described by Bliss et al. [2].

In control conditions, stimulation of the commissural pathway at 0.1 Hz evoked a negative population EPSP in the stratum radiatum of CA_1. As shown in Fig. 1A application of phorbol 12–13 diacetate (PDac) (500 μM, 5 min) rapidly produced a very large (over 100%) enhancement of the field EPSP. In the subsequent 20 min, the amplitude declined reaching a mean increase of 40% over the control (pre-drug) levels, the EPSP remained potentiated with little change for up to 2 h (the second hour is not shown). Similar observations were made in 8 experiments.

The effect of PDac on the content of endogenous glutamate and aspartate is shown in Fig. 1B, C which were obtained from the same experiment. Clearly, the protein kinase C activator produced a highly significant increase in the release of glutamate and aspartate. This increase was maximal in the first and second samples following PDac reaching values of 300% over the control (pre-drug) content. However, starting from the third sample (15 min after the application of PDac) the levels of glutamate and aspartate returned to control values.

Similar observations were made in 8 experiments. Thus as shown in Table I, the mean increase (for 8 cases) in the content of endogenous glutamate in the perfusate during the second sample following the application of PDac was of more than 500% over the control (pre-drug) values (range of 3- to 8-fold increase over the levels found in the control pre-drug samples).

Aspartate was also increased although the magnitude of this increase was smaller than that for glutamate (more that 300% over the control (pre-drug) values). Furthermore after this increase the levels of glutamate and aspartate in the following samples

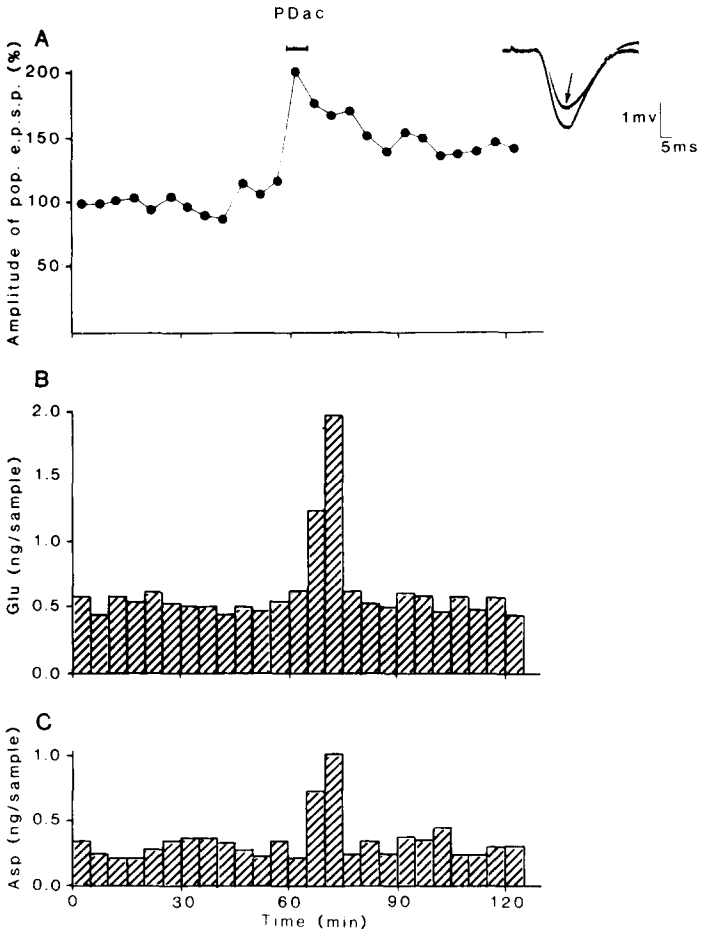

Fig. 1. Phorbol 12 13 diacetate (PDac) produces long-lasting potentiation of the field EPSP and a brief increase in the release of excitatory amino acids. A, B and C are from the same experiment. A: long-lasting potentiation of the field EPSP. The amplitude of the field EPSP evoked by a commissural stimulation at 0.1 Hz is indicated as a percent of the control (pre-drug) level. The photograph represents the field EPSP just before the application of PDac (arrow) and the potentiated EPSP 1 h after the application of the drug. One field EPSP was digitized every minute and 5 consecutive EPSPs were sampled to obtain the value shown in the figure, this mean EPSP therefore corresponds to one (5 min) sample used for HPLC (see below). Note that the application of PDac (500 μM, 5 min, arrow) produced a sustained enhancement of the EPSP (the EPSP was still enhanced 2 h after the application of PDac (not shown). B, C: release of glutamate (B) and aspartate (C) in the same experiment. The tissue was perfused with the push-pull device at a rate of 10 μl/min and 5-min samples were used for HPLC analysis. Note the stable release of endogenous amino acids in the control (pre-drug) situation. PDac (arrow) produced a brief (10 min) increase in the release (more than 300%) of glutamate and aspartate.

were not different from the control values, therefore, the enhancement of the field EPSP still present 2 h after PDac application, is not due to a persistent enhanced release of glutamate and aspartate.

In additional experiments, the application of smaller concentrations of PDac (50

TABLE I

THE GLUTAMATE AND ASPARTATE CONTENT IN EACH (5 min) SAMPLE FOLLOWING THE APPLICATION OF PHORBOL 12–13 DIACETATE (PDac) IS INDICATED AS A PERCENT OF THE CONTROL PRE-DRUG (15 min) PERIOD

The values of 8 experiments were pooled. t_1, t_2, t_3, t_4 refer to the order of the samples i.e. the first, second, third and fourth (5 min) sample after PDac; t_{10-12} refers to the mean value found in 3 consecutive samples taken 50–60 min after the application of PDac. Note that the enhancement of the release of glutamate and aspartate was only observed in samples 1 and 2 (*$P < 0.05$ Student's t-test).

	Glutamate	Aspartate
Control	100	100
t_0	144 ± 20	108 ± 23
t_1	260 ± 45*	240 ± 28*
t_2	560 ± 169*	298 ± 53*
t_3	85 ± 18	95 ± 10
t_4	88 ± 15	93 ± 18
t_{10-12}	88 ± 18	95 ± 10

μM or 100 μM ($n = 2$)) also produced a long-lasting potentiation of the field EPSP. The release of glutamate and aspartate was also increased (immediately after application of PDac), this increase was however of smaller magnitude (250% for aspartate and 300% for glutamate) than that seen with 500 μM of PDac. Furthermore, to test the effects of the solvent, in two additional cases, dimethyl sulfoxide (DMSO) was perfused for 5 min at the concentration used to dissolve PDac (2.5%). DMSO neither produced a potentiation of the EPSP nor significantly changed the release of glutamate and aspartate ($n = 2$, not shown).

The first conclusion which stems from the present study is that in the intact animal perfusion with PDac produces a significant increase in the release of glutamate and aspartate, the probable transmitters of the commissural pathway [8]. This is in agreement with studies in neuromuscular junctions employing quantal analysis [4, 9] and in other preparations [12, 13]. In a recent study, Malenka et al. [7] have also shown that PDac produces an increase in the release of glutamate in the hippocampal slice preparation, however the 2 experimental situations cannot be compared directly because these authors incubated their slices with high K^+ concentrations (50 mM) and exogenous aspartate (500 μM, to prevent the reuptake of glutamate). In addition, the basal release of amino acid in control conditions was not measured due to the poor sensitivity of the (microenzymatic) assay.

The present study also indicates that perfusion with PDac in the anaesthetized rats produces a typical long lasting enhancement of the commissural synaptic response in the hippocampus.

Our concomitant measurement of endogenous release of glutamate and aspartate indicates that this long-lasting enhancement of synaptic transmission cannot be explained by a persistant increase in the transmitter release since the levels of amino acids found in the perfusate 15 min to 2 h after the application of PDac, i.e. at a time when the field EPSP was still enhanced, were not significantly different from the con-

trol (pre-drug) levels. Therefore the long-lasting effect of PDac is not explicable exclusively by a presynaptic mechanism acting to produce a sustained enhancement of the release of transmitter.

Bliss et al. [2], using a similar experimental methodology, have recently provided evidence that a high-frequency train of electrical stimulation applied to the perforant path induced a LTP of the field EPSP in the molecular layer of the fascia dentata. This was associated with a sustained enhanced release of glutamate and aspartate, suggesting a presynaptic mechanism for the LTP. The discrepancy between the study of Bliss et al. and the present one may be due to differences in the systems investigated (fascia dentata vs stratum radiatum of CA_1) or the methodology used to induce the enhancement of the synaptic transmission (high-frequency train of electrical stimulation vs PDac). At any rate these observations raise the possibility that different mechanisms contribute to induce a long-lasting enhancement of synaptic transmission.

Financial support from Roussel-Uclaf was provided to L.A. We are grateful to Dr.R. Neuman for criticism of the manuscript and R. Amsellem for technical assistance.

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