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Tetanic stimulation affects the metabolism of phosphoinositides in hippocampal slices

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Tetanic but not low frequency stimulation of the perforant path in rat hippocampal slices results in changes in the metabolism of phosphoinositides and phosphatidic acid. The phosphorylation of other, non-inositol lipids was not affected by the high frequency stimulation. The observed changes in phosphoinositide metabolism are complex and biphasic, lasting at least 4 h after the termination of the tetanus. The present data support the notion that membrane phosphoinositides play a role in synaptic function.

Long-term potentiation (LTP), the enhancement of synaptic activity which remains for a long time after a brief period of electrical stimulation at high frequency[3,12], may be considered to constitute a model of a learning process at the synaptic level[23]. Several hypotheses have been advanced to explain LTP, implying both pre- and postsynaptic processes with an important role for calcium ions[10,13]. At the presynaptic level, LTP may be caused by an increase in the amount of transmitter released: at the postsynaptic level, LTP may be related to a modification of receptor properties, to a change in the spike-generating capacity of postsynaptic neurons or to a change in electrical properties of dendritic membranes[5,29]. The various possibilities are, of course, not necessarily mutually exclusive. For many years it has been known that changes in electrical activity of neurons are paralleled by changes in membrane phosphorylation. Heald[18] was the first to emphasize protein phosphorylation in this respect. Recently, two independent sets of experiments have revealed that tetanic stimulation of a monosynaptic pathway in rat hippocampal slices results in changes in the phosphorylation of proteins as determined by a post hoc in vitro phosphorylation assay[8,9,14,15,30]. Browning and co-workers[14,15] have shown that the α-subunit of pyruvate dehydrogenase — a key enzyme in mitochondrial energy metabolism, the activity of which is known to be regulated by phosphorylation — shows decreased phosphorylation after tetanic stimulation of the Schaffer collaterals. We have reported that LTP, elicited by stimulation of the perforant pathway, enhances the phosphorylation of an acidic protein (M_r 52,000; IEP 5.3) present in synaptic plasma membranes[7–9]. In addition, there is evidence that the phosphorylation of membrane proteins is related to the metabolism of a special class of membrane phospholipids, the (poly)phosphoinositides ((poly)PI)[19,21]. Several treatments, among them electrical stimulation of brain synaptosomes, give rise to an enhanced turnover of members of the phosphoinositide lipids[1,17,28]. Although previously the hydrolysis of PI was assumed to represent the first event after receptor activation[25,26], present studies emphasize the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) as the key event[27]. This notion is based among

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other evidence on the calcium-dependent degradation of prelabeled PIP_2 after α-adrenergic and cholinergic stimulation of the iris smooth muscle[2]. The PIP_2 breakdown is thought to be the result of phosphodiesterase activity stimulated by low concentrations of calcium[1,4,16].

In the present study we report that tetanic stimulation of the perforant pathway in rat hippocampal slices results in changes in the metabolism of phosphatidylinositol (PI), the (poly)phosphoinositides (PIP and PIP_2) and phosphatidic acid (PA).

Slices of rat hippocampus (300–400 μm) were prepared as described before[29] and incubated in a Krebs–Ringer buffer (NaCl 124 mM, KCl 5 mM, KH_2PO_4 1.24 mM, $MgSO_4$ 1.3 mM, $CaCl_2$ 2.0 mM, $NaHCO_3$ 26 mM, glucose 10 mM, pH 7.4) to which was added 20 μCi inorganic [^{32}P]phosphate. In this way phospholipids were labeled prior to electrical stimulation. It was established that after relatively short incubation times (10 and 30 min) phospholipid-bound radioactivity was recovered almost exclusively in PI, PIP, PIP_2 and PA (see Fig. 1). After a 10 min incubation with label, non-inositol phospholipids had incorporated approximately 4% of the total amount of radioactivity in lipids. In view of the differences in procedures, the absolute values of ^{32}P-incorporation as reported in the present paper cannot be compared to those obtained by Jolles et al.[20]. Yet, the relative distribution between label recovered in inositol- and non-inositol-containing phospholipids was found to be similar.

Subsequently, it was studied whether tetanic stimulation affects the metabolism of the (poly)PIs and PA. Hippocampal slices, prelabeled for 10 min as described above, were subjected to high frequency stimulation, applied to the perforant path (15 pulses/s for 15 s)[9]. Immediately after stimulation the slices were taken out of the stimulation chamber and homogenized in an ice-cold Tris buffer (Tris 10 mM, EDTA 1 mM; pH 7.4). Triplicate samples of this homogenate were extracted as described before[20] and phospholipids were separated using thin-layer chromatography. The amount of label, incorporated into each of them was determined by scraping the silica layer and subsequent liquid scintillation counting. The results are shown in Table I. The loss of label from PI was found to be 23%, whereas PIP_2 and PIP showed a loss of 16% and 20%, respectively. The disappearance of label from PA was not statistically significant.

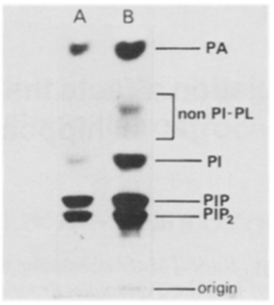

Fig. 1. Incorporation of radioactive phosphate into phospholipids after incubation of intact slices with [^{32}P]PO_4. A: 10 min incubation time; B: 30 min incubation time. Phospholipids were extracted, separated and visualized by autoradiography as described in ref. 21. PA, phosphatidic acid; non PI-PL, non-phosphoinositol phospholipids (phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine); PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate.

Another approach was used in order to follow changes in the (poly)PI metabolism induced by tetanic stimulation, over a longer period after the tetanus. Slices were preincubated in the stimulation chamber for 60 min without label, after which a tetanus was applied to the perforant path fibers[9,27]. The time between end-of-stimulus and start-of-labeling was varied from 2 min to 4 h; during these intervals, the slices stayed in the stimulation chamber. Previous experi-

TABLE I

Hydrolysis of radiolabeled phosphoinositides after tetanic stimulation of prelabeled rat hippocampal slices

n, number of slices; *, cpm (mean ± S.E.M.); △%, percentual difference from control slices; *P*, Student's *t*-test (two-tailed); o, not significant.

Phospholipid	*Controls (n = 5)*	*Stimulated (n = 5)*	△%	*P*
PI	891 ± 82*	688 ± 66	−23	$P < 0.05$
PIP	3949 ± 144	3153 ± 69	−20	$P < 0.01$
PIP_2	4740 ± 209	4003 ± 113	−16	$P < 0.05$
PA	2023 ± 174	1695 ± 131	−16	o

ments had shown that the viability of the slices kept under these conditions extended up to 6 h[7,29]. For every interval thus studied, unstimulated slices were kept in another stimulation chamber and labeled at the end of the interval. In this way every time point had its own unstimulated control and differences in labeling pattern are a function only of the time elapsed between treatment of the slice and labeling. After the incubation with labeled inorganic phosphate for 10 min, the slices were homogenized and the lipids extracted. The labeling of phospholipids in non-stimulated controls did not vary with the time between the end of pre-incubation and the start of labeling; in other words, the basic labeling pattern was constant irrespective of the time spent in the stimulation chamber (up to 4 h). In contrast, slices that did receive tetanic stimulation showed changes in the labeling of (poly)PI and PA in a time-dependent manner (Fig. 2). When the incubation with labeled phosphate was started 2 min after application of the tetanus, PIP, PIP_2 and PA showed a significant decrease in labeling, whereas PI did not. After 15 min there was a marked increase in PI labeling, contrasted by the virtual absence of an effect on the labeling of the other phospholipids. Starting 1 h after the tetanus, an enhanced incorporation of radioactive phosphate into PIP, PIP_2 and PA was observed which was maximal at 4 h after the tetanus. The labeling of PI appeared to decline over this period.

If the changes in labeling had been caused by an altered availability of γ-labeled ATP one would expect a general and uniform effect of the tetanus on phosphoinositides, other phospholipids and phosphoproteins. Instead, specific rather than general effects on

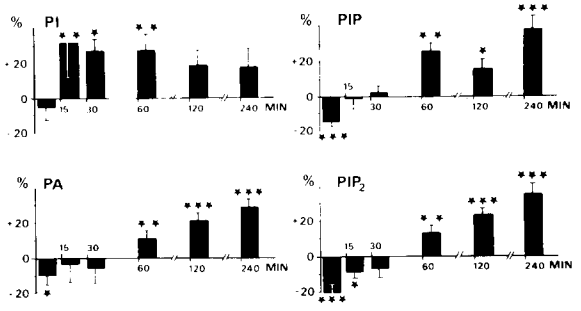

Fig. 2. Changes (percentage with respect to controls) in labeling of 4 phospholipids present in rat hippocampal slices after tetanic stimulation of the perforant path fibers. X-axis gives time interval between the end of the stimulation and the start of a 10 min incubation with labeled inorganic phosphate. Y-axis gives the change in incorporation of labeled phosphate in percentages; bars indicate mean ± S.E.M. Statistical evaluation consisted of one-way analysis of variance followed by the Student's *t*-test (two-tailed). Asterisks indicate the *P* value: one, $P < 0.05$, two, $P < 0.01$; and three, $P < 0.001$. Abbreviations: see legend to Fig. 1.

protein phosphorylation have been reported, arguing against an effect on supply of ATP to the relevant kinases[8,9,14,15,30]. Likewise it was found that the tetanus does not affect the incorporation of phosphate into the non-inositide phospholipids under our experimental conditions.

The exact relation between the development of LTP and the changes in (poly)PI metabolism is not clear. However, we were able to demonstrate that the changes are dependent on the frequency of the stimulation used: when a stimulation with low frequency was used, which was previously shown not to produce LTP[8], no changes in labeling of the inositol phospholipids were observed. In this experiment hippocampal slices received 225 pulses in the perforant

TABLE II

Incorporation of radioactive phosphate into phosphoinositides and phosphatidic acid at various time periods after a low or high frequency electrical stimulation of rat hippocampal slices

LO-STIM, low stimulation; HI-STIM, high stimulation (n = 5); △%, percentual difference from control slices; *P*, Student's *t*-test (two-tailed); o, not significant; $*P < 0.01$; $**P < 0.001$.

	2 min[a]				*60 min*				*240 min*			
	LO-STIM		*HI-STIM*		*LO-STIM*		*HI-STIM*		*LO-STIM*		*HI-STIM*	
	△%	*P*	△%	*P*	△%	*P*	△%	*P*	△%	*P*	△%	*P*
PI	+5	o	−5	o	+15	o	+28	*	−12	o	+18	o
PIP	0	o	−14	**	+1	o	+25	*	−10	o	+37	**
PIP_2	−8	o	−20	**	−0	o	+12	*	−5	o	+35	**
PA	−3	o	−9	o	−9	o	+11	*	−7	o	+29	**

[a] Time after electrical stimulation.

path fibers. Two frequencies were applied, one pulse per 4 s, during 900 s (LO-STIM), or 15 pulses per s during 15 s (HI-STIM, same frequency as used above). After stimulation the slices were kept in the incubation chamber for 2, 60 or 240 min. Subsequently, they were incubated with 30 μCi [^{32}P]phosphate and treated as described above. Again, incorporation of label into the difference inositol lipids and PA was determined quantitatively. The HI-STIM results (shown in Table II) replicate largely what was found earlier (see Fig. 2). However, low frequency stimulation (Table II, LO-STIM) produced no significant effects on labeling of any of the lipids studied.

The presently reported experiments strongly suggest that the metabolism of phosphoinositides in hippocampal slices is influenced by tetanic stimulation. The data obtained with the stimulation after prelabeling of the phospholipids were taken to indicate that the electrical stimulation enhances the hydrolysis of PIP_2, PIP and PI. Also the series of experiments using labeling of slices after tetanic stimulation imply that shortly after the tetanus there is a high turnover of PI, PIP and PIP_2. In fact, the enhanced PI labeling at 15, 30 and 60 min after the tetanus may reflect a compensatory resynthesis after a preceding massive PI hydrolysis.

The biphasic changes in labeling of the (poly)PI closely parallel the time course seen in the development of the LTP as described earlier; immediately after the tetanic stimulation there is a depression in responsiveness to the test stimulus, followed by a slowly developing potentiation which reaches a maximum after 1–2 h[29].

It may be that the changes in (poly)PI metabolism as reported here are related to intracellular calcium mobilization[1]. Other studies have shown that changes in intracellular calcium concentration either pre- or postsynaptic are related to or causative in the occurrence of LTP[6,10,15,22,24]. Therefore, it may be that the present experiments have revealed membrane events which are part of the biochemical changes observed after tetanic stimulation.

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