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Dopamine-Induced Changes in Protein Phosphorylation and Polyphosphoinositide Metabolism in Rat Hippocampus

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Effects of dopamine (DA) on endogenous phosphorylation of hippocampal proteins and polyphosphoinositides were studied in subcellular fractions from a crude mitocondrial/synaptosomal preparation. DA induced a concentration-dependent decrease in the in vitro phosphorylation of the protein B-50 (—22.1% at 10^{-5} M DA), whereas no changes were found in phosphoproteins in other subcellular fractions. Treatment of hippocampal slices with 5×10^{-4} M DA resulted in a 45.8% increase in post hoc phosphorylation of B-50 in SPM and it affected post hoc phosphorylation of several proteins in a cytosolic fraction. In vitro phosphorylation of SPM with DA (5×10^{-4} M) increased endogenous TPI phosphorylation (+ 51.6%), whereas treatment of slices with DA (5×10^{-4} M) resulted in a 39.4% decrease in post hoc TPI phosphorylation. This decrease could be blocked by haloperidol. Significant changes induced by DA (5×10^{-4} M) were also found in ^{32}P-incorporation into PA (in vitro: —32.4% and post hoc: + 39.3%), but were not found in DPI labeling. The data provide evidence for DA-induced changes in phosphorylation of proteins and polyphosphoinositides in rat hippocampal SPM.

INTRODUCTION

The phosphorylation of membrane components is thought to play an important role in the regulation of neuronal functions[20,42,47]. Both phosphorylated proteins[21] and a special class of phospholipids[36,37], the (poly)phosphoinositides (polyPI), have been implicated in the regulation of synaptic transmission and membrane ion permeability. The enzymes responsible for the protein phosphorylation reactions are largely found in cellular fractions enriched in synaptic plasma membranes (SPM)[21,23]. Endogenous phosphorylation in SPM may be regulated directly by neurotransmitters (acetylcholine[22]) and neuropeptides (ACTH[48]; enkephalins[2,11]), or indirectly by transmitters known to affect intracellular cAMP levels (noradrenaline and dopamine[21,42,46]; serotonin[14]).

Transmitter–receptor interaction may also be involved in the regulation of macromolecular synthesis[24,41]. Electrophysiological[18], autoradiographic[45], behavioral[19] and biochemical[5,6,25,43] studies point to the existence of a dopaminergic transmitter system or dopamine (DA)-sensitive structures in the hippocampus. Dopaminergic agonists increase the fucosylation of glucoproteins in hippocampus in vivo[30] and in hippocampal slices[29]. Similar results are obtained with slices of the corpus striatum[33], a brain region rich in dopaminergic synapses and receptor sites.

The hippocampal slice has proven to be a suitable model, as it is electrophysiologically well characterized and it appears to retain the biochemical and electrophysiological characteristics of the in vivo system[35]. In the hippocampal slice it was shown that repetitive electrical stimulation of intact monosynaptic neuronal circuits, which is known to produce long-term potentiation (LTP), induces changes in the phosphorylation state of specific synaptic phosphoproteins[3,8–10,32] and in polyPI metabolism[1]. Whatever the neurochemical mechanism underlying LTP, it is generally accepted that LTP is a reflection of an enhanced synaptic efficacy[7].

Thus, in the hippocampus both phosphorylation of membrane components and fucosylation of glycoproteins appear to be related to neurotransmitter action. Since DA induces changes in fucosylation of hippocampal glycoproteins, we were interested in possible concomitant changes in phosphorylation of membrane proteins and lipids. In this study we describe

the effects of DA on in vitro phosphorylation of hippocampal SPM and on post hoc phosphorylation of membranes isolated from treated and untreated hippocampal slices.

MATERIALS AND METHODS

Hippocampal dissection and slice preparation

Male rats (140–150 g) of an inbred Wistar strain (TNO, Zeist, The Netherlands) were used. After decapitation the brain was quickly removed and the hippocampi were dissected within 2 min after death. They were either immersed in ice-cold 0.32 M sucrose for subsequent tissue fractionation (see below) or were kept at room temperature in a Krebs–Ringer buffer (KRB) (NaCl 125 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, equilibrated with a gas mixture of 5% CO_2 and 95% O_2 prior to slice preparation. Slices of 300–400 μm thickness were cut by hand perpendicular to the long axis with a special multiple slice cutting device holding 4 razor blades.

Dopamine incubations

In each experiment slices cut from 8 rats were divided into groups of 12 per test tube and preincubated for 60 min in 1.8 ml KRB at 36 °C in a shaking water bath under constant gassing. The incubation was started by the addition of DA (Koch Light Lab. Ltd.; in KRB, final concentration 5×10^{-4} M) or KRB to the tubes. In some experiments haloperidol (Janssen Pharmaceutica; in KRB, final concentration 10^{-4} M) was added 15 min prior to the DA. The incubation volume was 2.0 ml. Incubations were stopped after 5 min by washing the slices twice in 2.0 ml ice-cold 0.32 M sucrose solution.

Tissue fractionation

From 8 freshly dissected hippocampi or from 12 pooled slices a crude mitochondrial/synaptosomal fraction (P2) was prepared[48]. This P2 pellet was osmotically shocked in 9 vols. distilled water for 15 min and centrifuged at 10,000 *g* for 20 min to spin down mitochondria and unlysed material.

The supernatant was subsequently layered on top of a small discontinuous sucrose gradient (0.4 M: 1.3 ml; 1.0 M: 2.2 ml) and centrifuged at 100,000 *g* for 80 min[12,13]. Material floating on top of the 1.0 M layer was collected, diluted with acetate buffer (sodium acetate 10 mM, magnesium acetate 10 mM, calcium acetate 0.1 mM, pH 6.5) and a fraction enriched in light synaptosomal plasma membranes (SPM) was collected by centrifugation (100,000 *g* for 20 min). The 0.4 M sucrose fraction was diluted with acetate buffer and centrifuged at 100,000 *g* for 20 min to obtain a synaptic vesicle-enriched fraction. The fraction remaining on top of the gradient was called cytosolic fraction.

Phosphorylation assay

Endogenous phosphorylating activity was assayed as described earlier[31,48]. Briefly, incubations were performed under the following conditions: 7.5 μM ATP, 2-4 μCi [γ-^{32}P]ATP (approx. 3000 Ci/mmol, Amersham, U.K.), 10–20 μl subcellular hippocampal fraction (containing 10–15 μg protein), acetate buffer, pH 6.5, final volume 25 μl. Subcellular fractions were preincubated for 6 min at 30 °C and the incubations (15 s) were started by the addition of ATP. In some experiments haloperidol (final concentration 10^{-4} M) was added at the beginning of the preincubation and DA (final concentration 5×10^{-4} M) one minute later. Protein phosphorylation was stopped by adding 12.5 μl of a denaturing solution, resulting in final concentrations of 62.5 mM Tris/HCl, 2% SDS, 10% glycerol, 0.001% bromophenol blue, 5% 2-mercaptoethanol. Lipid phosphorylation was stopped by adding 2 ml ice-cold chloroform/methanol/12 N HCl (200:100:0.75, by vol.)[44]. Protein was determined according to Lowry et al.[34].

Qualitative and quantitative analysis

Denatured proteins in subcellular fractions were separated according to molecular weight on polyacrylamide slab gels, as described by Zwiers et al.[48]. Slab gels (1.3 mm thick) consisted of a running gel (11% acrylamide, 9 cm) and a stacking gel (3% acrylamide, 2 cm). Routinely, 37.5 μl samples were applied containing 10–15 μg protein. Electrophoresis was carried out at room temperature, using 30 mA (100–200 V) per gel until the tracking dye reached the bottom of the gel (about 3 h). The proteins were stained for 10 min in 0.1% Fast Green in destaining solution (acetic acid:water:methanol, 10:40:50, by vol.), destained overnight and dried.

Quantitative autoradiography was performed using Kodak Royal X-O-mat film (exposure times be-

tween 1 and 4 days) to visualize radioactivity. Radioactivity was quantified by making a linear scan of the autoradiograms using a Zeiss PMQII, coupled to a Zeiss scanning densitometer (slit width 0.03 mm, wavelength 550 nm) and peak heights above background were measured[2].

After stopping the phosphorylation lipids were extracted according to Jolles et al.[27]. Briefly, carrier polyPI were added and a biphasic system was obtained by the addition of 0.375 ml of 0.6 N HCL. The upper phase was removed and the lower phase washed with 1 ml chloroform/methanol/0.6 M HCl (3:48:47, by vol.). The resulting lower phase was dried under N_2 and redissolved in chloroform/methanol/water (75:25:2, by vol.). Samples were taken for the determination of radioactivity incorporated into total phospholipids.

Phospholipids were separated by high performance thin-layer chromatography (HPTLC; layer thickness 25 μm, Merck). Before use, plates were impregnated with potassium oxalate (1%) and activated for 15 min at 110 °C. The lipid extract was applied to the plate and lipids were separated with chloroform/aceton/methanol/glacial acetic acid/water (40:15:13:12:8, by vol.). This procedure yields quantitative separation of labeled lipids. They were visualized with iodine vapour and ^{32}P-labeled spots were detected by autoradiography on Kodak Royal X-Omat film (10–20 h). The spots were scraped from the plates and counted for radioactivity in a liquid scintillation spectrometer.

All operations (slice incubation, tissue fractionation, phosphorylation, lipid extraction and HPTLC) were performed on the same day.

RESULTS

DA effects on protein phosphorylation

In the first series of experiments hippocampi from 8 rats were pooled to prepare a mitochondrial/synaptosomal fraction (P2), which was subsequently subfractionated into a cytosolic, a vesicle-enriched and a light SPM fraction. Each fraction was phosphorylated with or without 10^{-5} M DA. Autoradiograms of the phosphorylated fractions are shown in Fig. 1. In the SPM fraction (Table I) DA induced a significant

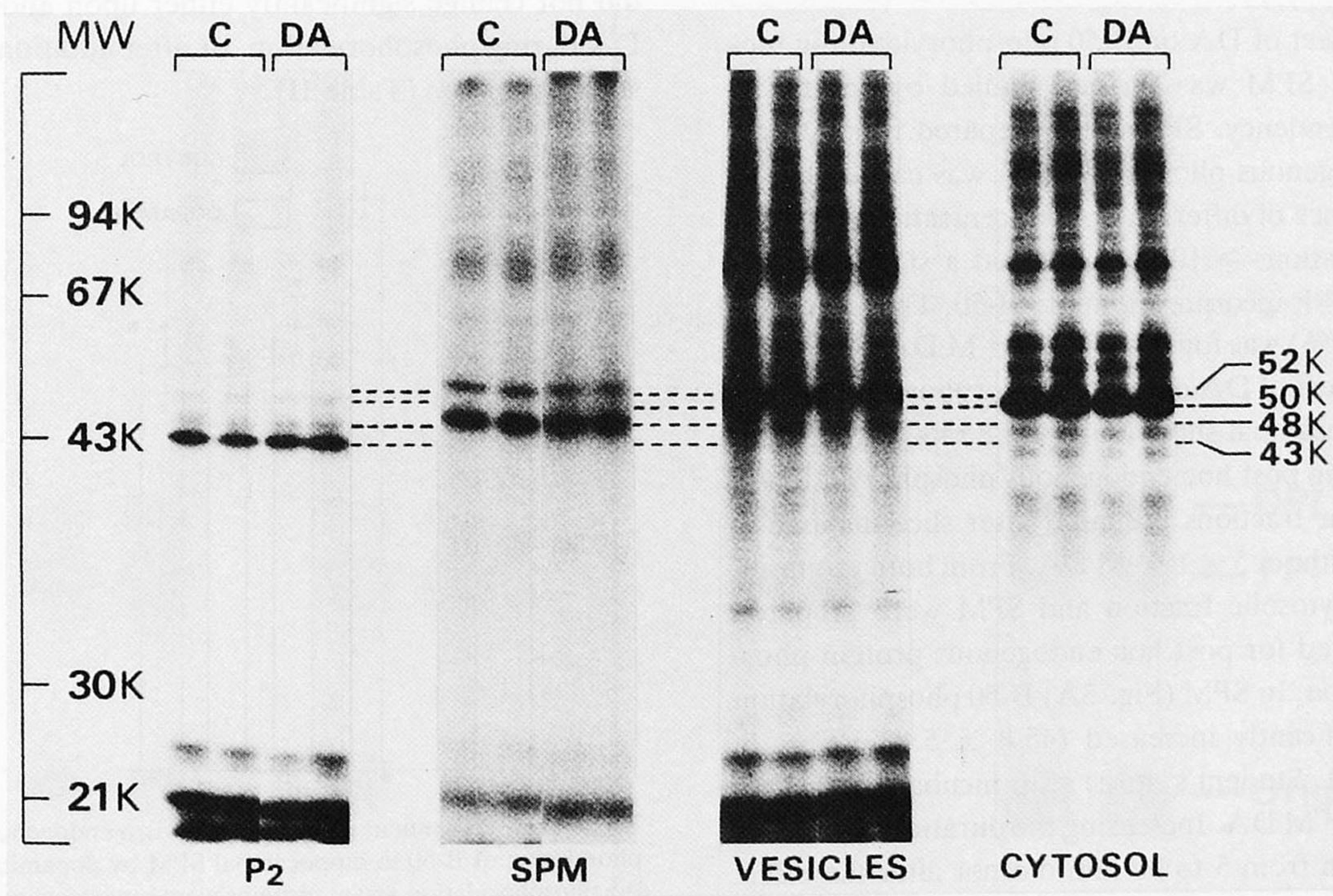

Fig. 1. Autoradiograms showing the effect of dopamine on in vitro endogenous phosphorylation of rat hippocampal proteins in subcellular synaptosomal fractions. C, control; DA, 10^{-5} M dopamine; P2, crude mitochondrial/synaptosomal fraction; SPM, synaptosomal plasma membrane-enriched fraction; VESICLES, vesicle-enriched fraction; CYTOSOL, cytosolic fraction. Positions of molecular weight markers (MW) are indicated at the left. The apparent M_r's of several major phosphoproteins are indicated at the right.

TABLE I

Effect of 10^{-5} M dopamine on in vitro endogenous phosphorylation of hippocampal proteins in subcellular fractions of a crude mitochondrial/synaptosomal preparation (P2)

Protein band (apparent molecular weight)	*Subcellular fraction*		
	P2	*SPM*	*Vesicles*
52 K	n.d.	—2.2 ± 4.1+ (11)	+3.8 ± 1.4 (6)
50 K	+7.3 ± 3.6 (6)	n.d.	n.d.
48 K	n.d.	—22.1 ± 2.3* (11)	+4.3 ± 4.7 (6)
43 K	+3.1 ± 3.4 (7)	n.d.	n.d.

+ Percentage change of ^{32}P-incorporation into protein band as compared to control ± S.E.M.; numbers in brackets indicate the number of determinations. (n.d.), not detectable.

* Significantly different from control, $P < 0.01$ (Student's *t*-test).

decrease (—22.1%) in ^{32}P-incorporation into a protein band with an apparent M_r of 48 K, identified as B-50[50]. No significant changes in ^{32}P-incorporation due to DA treatment were found in other major phosphoproteins with apparent M_r's between 40 and 60 K.

The effect of DA on B-50 phosphorylation in hippocampal SPM was further studied by testing its dose-dependency. SPM were prepared from 10 rats and endogenous phosphorylation was carried out in the presence of different DA concentrations (Fig. 2). Concentrations $\geqslant 10^{-5}$ M induced a significant decrease in ^{32}P-incorporation into B-50. The largest effect (—29%) was found at 5×10^{-4} M DA.

The effect of DA on protein phosphorylation in intact hippocampal slices (cut from 8 rats) was studied by assaying post hoc endogenous phosphorylation in subcellular fractions prepared after slice incubation with or without 5×10^{-4} M DA. From both groups of slices a cytosolic fraction and SPM were prepared and assayed for post hoc endogenous protein phosphorylation. In SPM (Fig. 3A) B-50 phosphorylation was significantly increased (45.8 ± 5.8%; n = 6, $P < 0.001$, Student's *t*-test) after incubation of slices in 5×10^{-4} M DA. Increasing the duration of the slice incubation from 5 to 30 min did not alter the effect (results not shown). In the cytosolic fraction (Fig. 3B) DA increased post hoc phosphorylation of 3 protein bands with apparent M_r's of 80 K, 56 K and 45 K, and decreased post hoc phosphorylation of 2 other protein bands with apparent M_r's of 65 K and 50 K.

DA effects on polyPI metabolism

Previously it was demonstrated that the state of B-50 phosphorylation in a semi-purified B-50 preparation[26] as well as in SPM[40] is closely related to the activity of endogenous DPI-kinase, the enzyme phosphorylating DPI to TPI. Therefore, we studied the effects of DA on polyPI metabolism in similar hippocampal SPM fractions as used for protein phosphorylation studies. A typical autoradiogram of HPTLC separation of phospholipids extracted from SPM (Fig. 4) shows 3 radioactive spots representing TPI, DPI and PA (hardly visible on photographs). In vitro endogenous phosphorylation of SPM (Table II) in the presence of 5×10^{-4} M DA resulted in a 51.6% increase in TPI phosphorylation, whereas incubation of slices in 5×10^{-4} M DA resulted in a 39.4% decrease in post hoc TPI phosphorylation. Significant changes due to addition of DA were also found in ^{32}P-incorporation into PA (in vitro: —32.4% and post hoc: +39.3%). The incorporation of label into DPI did not change significantly either upon addition of DA during phosphorylation, or after addition during slice incubation (Table II).

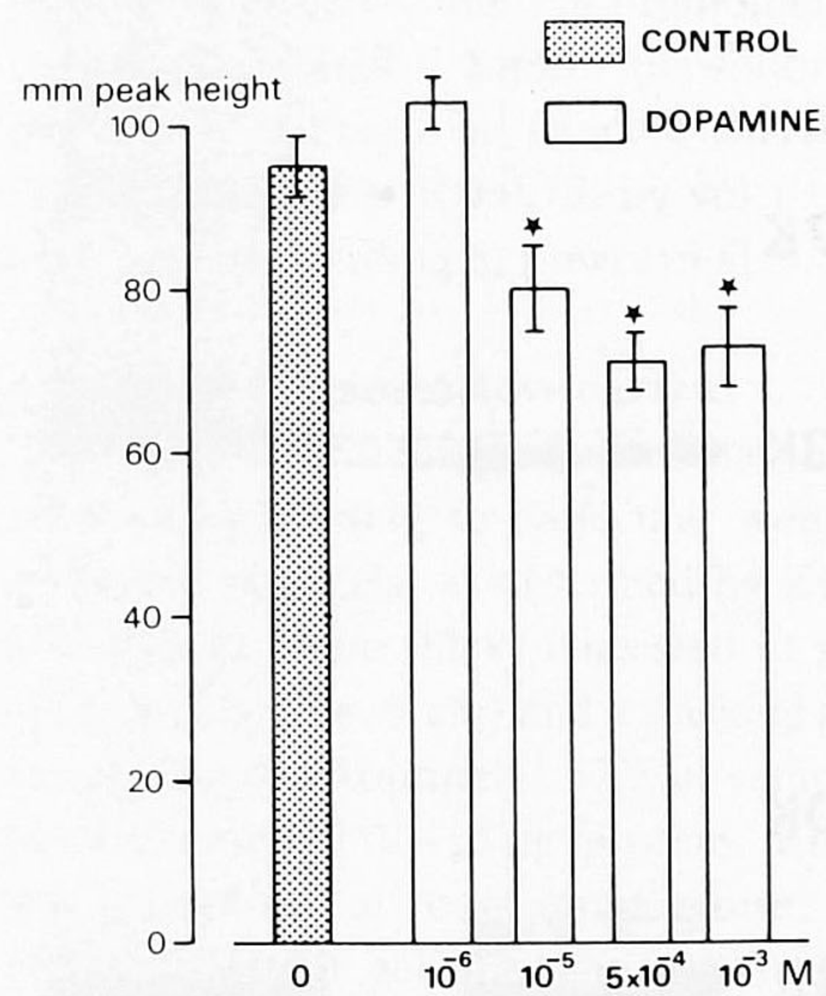

Fig. 2. Dose-dependent inhibition of in vitro endogenous phosphorylation of B-50 in hippocampal SPM by dopamine. After the phosphorylation assay, proteins were separated on slab gels and the gels were subjected to autoradiography. ^{32}P-incorporation into the B-50 band was measured as peak height above background by making scans of the autoradiograms (see Material and Methods). Bars indicate S.E.M., n = 4; *, $P < 0.01$ (Student's *t*-test).

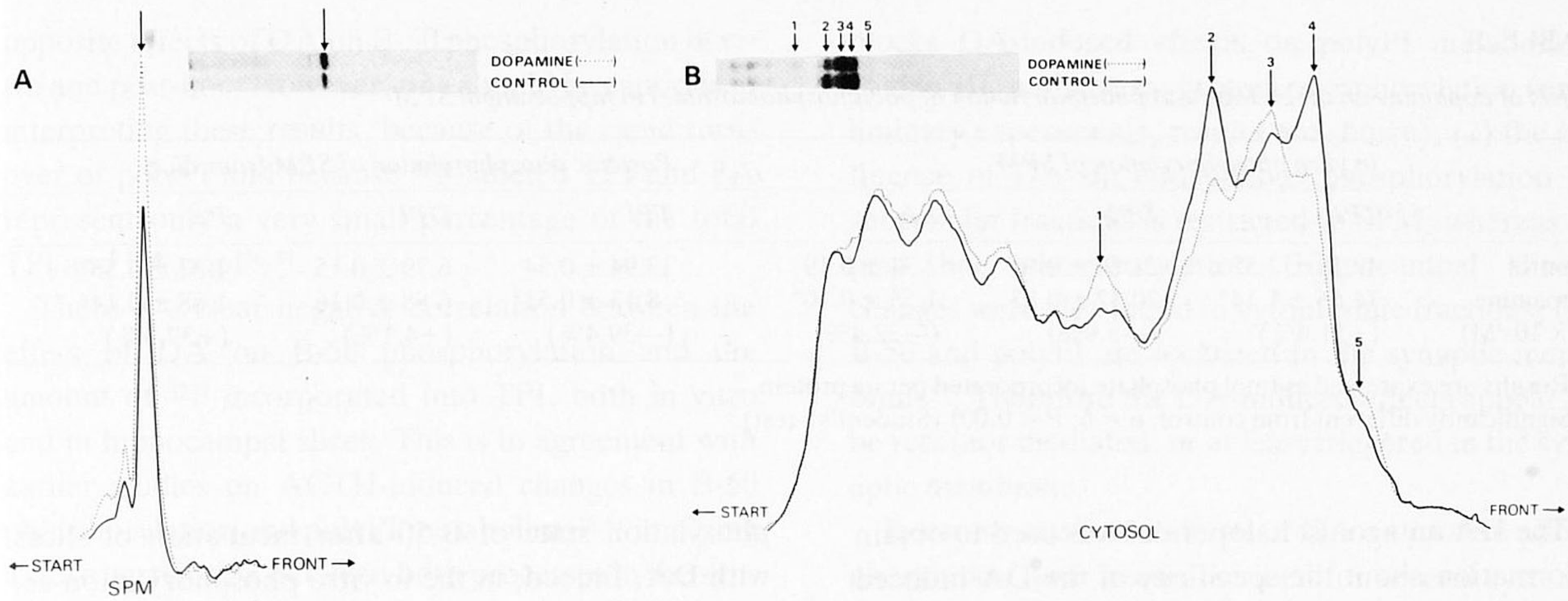

Fig. 3. Effect of dopamine treatment of hippocampal slices on post hoc endogenous protein phosphorylation in subcellular synaptosomal fractions. Slices were incubated for 5 min with (. . .) or without (——) 5×10^{-4} M dopamine. After subcellular fractionation and phosphorylation, proteins were separated and the slab gels were subjected to autoradiography (see Material and Methods). Densitometric scans of tracks in the autoradiograms (shown in the inserts) are presented. A: SPM, the arrow indicates protein B-50. B: cytosol, the arrows (and numbers) indicate phosphoproteins affected by dopamine treatment.

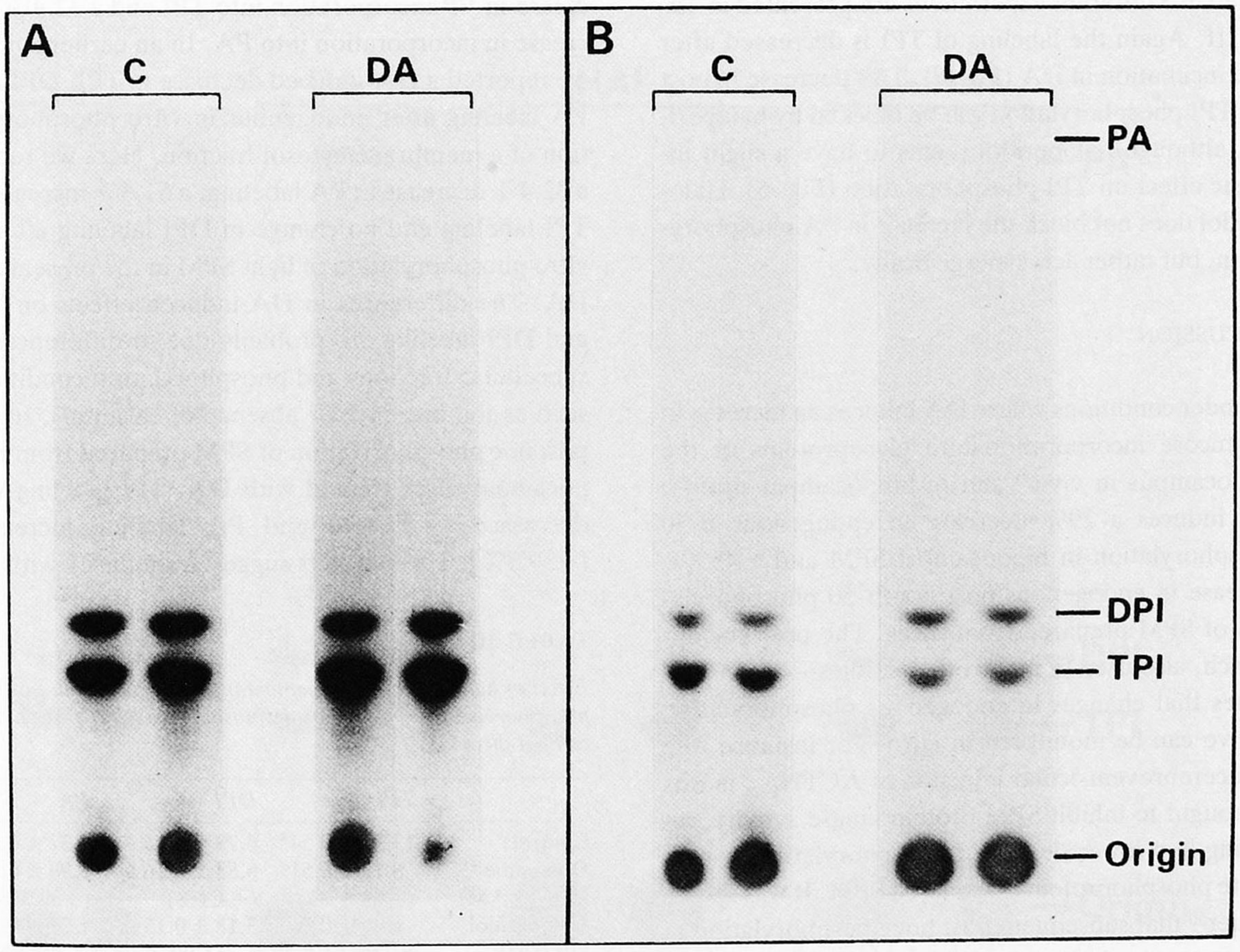

Fig. 4. Autoradiograms showing the effect of dopamine on the incorporation of ^{32}P into polyphosphoinositides in hippocampal SPM. A: in vitro endogenous phosphorylation of SPM with or without addition of dopamine 5 min prior to phosphorylation. B: post hoc phosphorylation of SPM prepared from slices incubated for 5 min with or without dopamine. After phosphorylation phospholipids were extracted, separated and visualized by autoradiography (see Material and Methods). C, control; DA, 5×10^{-4} M dopamine.

TABLE II

Effect of dopamine on the endogenous phosphorylation of polyphosphoinositides in hippocampal SPM

	In vitro phosphorylation of SPM			*Post hoc phosphorylation of SPM from slices*		
	TPI	*DPI*	*PA*	*TPI*	*DPI*	*PA*
Control	49.23 ± 2.57+	21.80 ± 0.91	2.31 ± 0.19	13.94 ± 0.54	6.79 ± 0.15	1.37 ± 0.55
Dopamine	74.66 ± 1.14*	20.47 ± 0.83	1.55 ± 0.10*	8.13 ± 0.51*	6.88 ± 0.16	1.88 ± 0.14*
(5×10^{-4}M)	(+51.6%)	(—5.9%)	(—32.4%)	(—39.4%)	(+4.1%)	(+39.3%)

+ Results are expressed as fmol phosphate incorporated per μg protein.
* Significantly different from control, n = 6; $P < 0.001$ (Student's t-test).

The DA antagonist haloperidol was used to obtain information about the specificity of the DA-induced changes in polyPI metabolism. Hippocampal slices of 8 rats were divided into 4 groups and incubated with or without DA and/or haloperidol. From each group SPM were prepared and assayed for post hoc lipid phosphorylation. Quantitative analysis of data obtained in 3 different experiments are presented in Table III. Again the labeling of TPI is decreased after slice incubation in DA (Fig. 5). This decrease in post hoc TPI phosphorylation can be blocked by haloperidol, although haloperidol seems to have a slight intrinsic effect on TPI phosphorylation (Fig. 5). Haloperidol does not block the increase in PA phosphorylation, but rather acts synergistically.

DISCUSSION

Under conditions where DA induces an increase in [^{3}H]fucose incorporation into glycoproteins of the hippocampus in vivo[30] and in hippocampal slices[29], DA induces a 29% decrease in endogenous B-50 phosphorylation in hippocampal SPM and a 45.8% increase in endogenous post hoc B-50 phosphorylation of SPM prepared from slices. The post hoc approach, also used in many other studies[2,3,8,9,15,49], assumes that changes in endogenous phosphorylation in vivo can be monitored in vitro. For instance, the intracerebroventricular injection of $ACTH_{1-24}$ in rats is thought to inhibit SPM protein kinase activity, resulting in a lower degree of phosphorylation of substrate phosphoproteins in isolated SPM. It was shown earlier[50] that subsequent post hoc phosphorylation of substrate proteins resulted in an increased incorporation of [γ-^{32}P]ATP. Likewise, in the present experiments the increase in endogenous post hoc phosphorylation of B-50 could reflect a decrease in the phosphorylation state of B-50 after incubation of slices with DA. Indeed, in the in vitro phosphorylation assay DA induced a decrease in B-50 phosphorylation.

In addition to the effects of DA on B-50 phosphorylation, DA treatment also affects polyPI metabolism. Endogenous phosphorylation of SPM in vitro in the presence of 5×10^{-4} M DA results in a 51.6% increase in ^{32}P-incorporation into TPI and a 32.4% decrease in incorporation into PA. In an earlier study[28] we reported a DA-induced decrease in TPI, DPI and PA labeling after endogenous in vitro phosphorylation of a membrane/cytosol fraction. Here we report a 32.4% decrease in PA labeling, a 51.6% increase in TPI labeling and no change in DPI labeling after in vitro phosphorylation of light SPM in the presence of DA. The differences in DA-induced effects on TPI and DPI labeling are probably due to differences in subcellular fractions and phosphorylation conditions such as the presence or absence of calcium[27]. In the post hoc phosphorylation of SPM prepared from hippocampal slices treated with DA, TPI labeling was decreased (—39.4%) and PA labeling increased (+39.3%). These results suggest a similarity with the

TABLE III

Effect of haloperidol on dopamine-induced changes in post hoc phosphorylation of polyphosphoinositides in SPM from hippocampal slices

	TPI	*DPI*	*PA*
Control	13.94 ± 0.54*	6.79 ± 0.15	1.37 ± 0.55
Dopamine	8.13 ± 0.51	6.88 ± 0.16	1.88 ± 0.14
(5×10^{-4} M)	(—39.4%)	(+4.1%)	(+39.3%)
Haloperidol	9.69 ± 0.56	3.18 ± 0.15	1.21 ± 0.11
(10^{-4} M)	(—27.9%)	(—51.9%)	(—10.4%)
Dopamine +	13.16 ± 0.37	5.63 ± 0.18	2.57 ± 0.03
haloperidol	(—1.9%)	(—14.8%)	(+90.4%)

* Results are expressed as fmol phosphate incorporated per μg protein (n = 4).

opposite effects of DA on B-50 phosphorylation in vitro and post-hoc. However, one should be cautious in interpreting these results, because of the rapid turnover of polyPI and because ^{32}P-labeled TPI and PA represent only a very small percentage of the total TPI and PA pool[36,37].

There is a clear negative correlation between the effect of DA on B-50 phosphorylation and the amount of ^{32}P incorporated into TPI, both in vitro and in hippocampal slices. This is in agreement with earlier studies on ACTH-induced changes in B-50 phosphorylation and polyPI metabolism[26,51] implying a close correlation between both processes in the synaptic membrane.

Concerning the mechanism of action of DA on SPM phosphorylation no final conclusion can be drawn. However, available evidence points to a receptor-mediated phenomenon: (1) haloperidol blocks DA-induced effects on polyPI metabolism (Table III) and also on protein phosphorylation (preliminary experiments, results not shown); (2) the influence of DA on endogenous phosphorylation in subcellular fractions is restricted to SPM, whereas in post hoc phosphorylation (hippocampal slices) changes were also found in cytoplasmic fractions; (3) B-50 and polyPI are localized in the synaptic membrane[51]. Therefore the DA-induced effects appear to be receptor mediated, or at least triggered in the synaptic membrane.

Receptor-mediated effects of DA on protein phosphorylation have been described for several systems including protein I in bovine superior cervical ganglia[38] and rat neostriatal membranes[16]. Moreover, there is accumulating evidence for the existence of DA receptors in the hippocampus[5,6] and for a role of DA in this brain region as neurotransmitter[4,18,43].

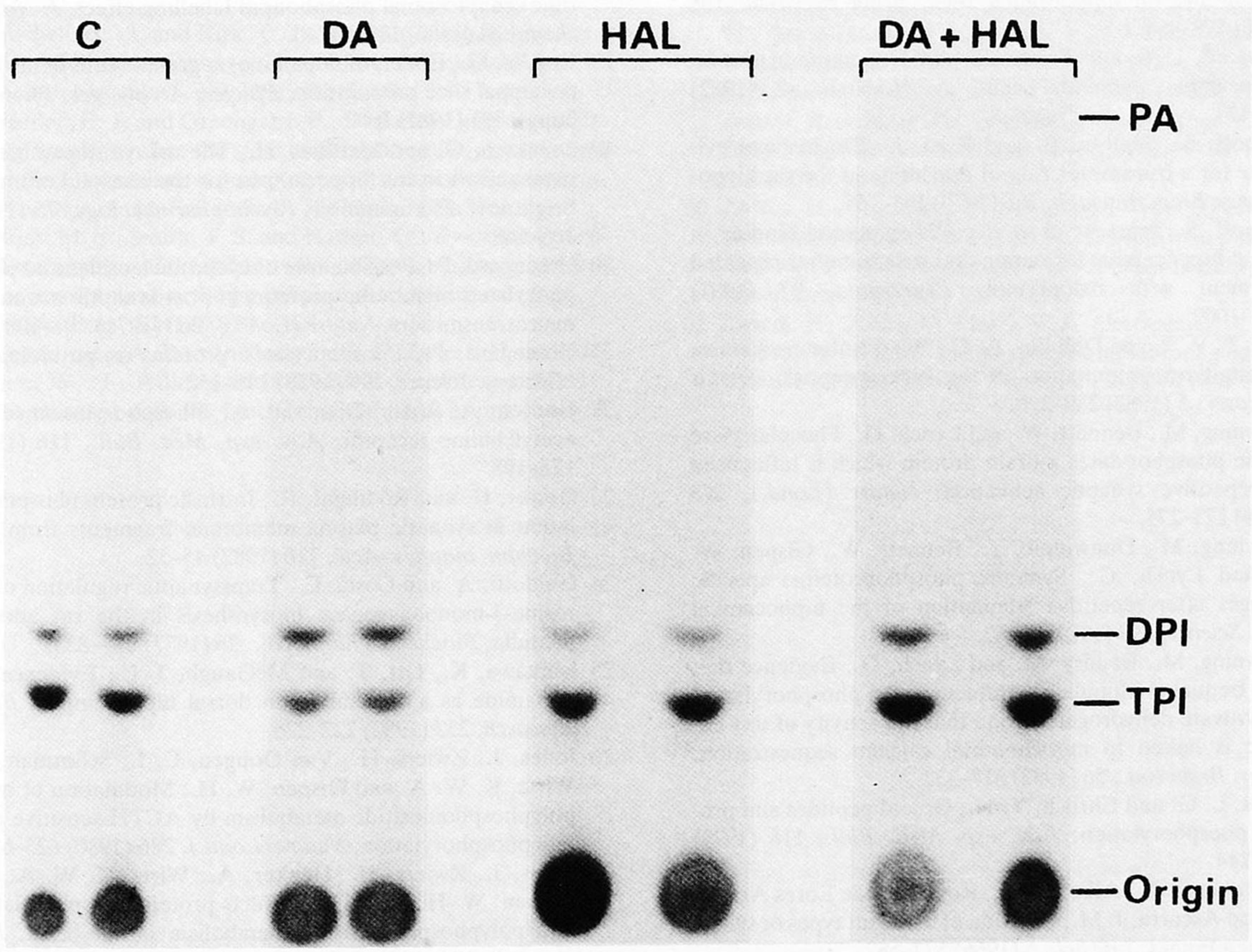

Fig. 5. Effect of haloperidol on dopamine-induced changes in post hoc phosphorylation of polyphosphoinositides in SPM prepared from hippocampal slices. Haloperidol (10^{-4} M) was added 15 min prior to a 5 min incubation of the slices with or without dopamine (5×10^{-4} M). Post hoc phosphorylation of SPM was followed by lipid extraction and separation, and polyphosphoinositides were visualized by autoradiography (see Material and Methods). C, control; DA, dopamine; HAL, haloperidol.

To date little is known about receptor-mediated effects of DA on polyPI metabolism. Treatment of intact cells or synaptosomes with muscarinic cholinergic[17] and α-adrenergic agonists[39] has been reported to affect polyPI metabolism. DA-induced receptor-mediated changes in glycoprotein synthesis have been described under similar conditions as used in this study. Since both phosphorylation of protein and lipid and glycoprotein synthesis have been implicated in mechanisms underlying changes in neuronal plasticity, it is tempting to speculate about a relation between the DA-induced changes in both phenomena.

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