Peptides, Vol. 3, pp. 709–714, 1982. Printed in the U.S.A.

Polyphosphoinositide Metabolism in Rat Brain: Effects of Neuropeptides, Neurotransmitters and Cyclic Nucleotides

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Received 7 June 1982

JOLLES, J., C. J. VAN DONGEN, J. TEN HAAF AND W. H. GISPEN. *Polyphosphoinositide metabolism in rat brain: Effects of neuropeptides, neurotransmitters and cyclic nucleotides*. PEPTIDES 3(5) 709–714, 1982.—This study describes effects of various peptides, neurotransmitters and cyclic nucleotides on brain polyphosphoinositide metabolism *in vitro*. The interconversion of the polyanionic inositol phospholipids was studied by incubation of a lysed crude mitochondrial/synaptosomal fraction with [γ-^{32}P]-ATP. The reference peptide $ACTH_{1-24}$ stimulated the formation of radiolabelled phosphatidylinositol 4,5-diphosphate (TPI) and inhibited that of phosphatidic acid (PA). Substance P inhibited both TPI and PA labelling, whereas β-endorphin inhibited that of PA without any effect on TPI. Morphine had no effect at any concentration tested, whereas high concentrations of naloxone inhibited the labelling of both PA and TPI. Naloxone did not counteract the effects of $ACTH_{1-24}$. The other peptides tested (lysine^8-vasopressin and angiotensin II) were without any effect. Under the conditions used, adrenaline, noradrenaline and acetylcholine did not affect the labelling of the (poly)phosphoinositides. Both dopamine and serotonin, however, dose-dependently inhibited the formation of radiolabelled TPI and PA. Low concentrations of cAMP stimulated TPI, but higher concentrations had an overall inhibitory effect on the labelling of TPI, PA and especially phosphatidylinositol 4-phosphate (DPI). The cyclic nucleotide did not mediate or counteract the effects of ACTH, and cGMP was without any effect. These results are discussed in the light of current ideas on the mechanism of action of neuropeptides.

ACTH β-Endorphin Polyphosphoinositides Phosphatidylinositol Substance P Opioids Cyclic nucleotides Neurotransmitters

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NEUROPEPTIDES, which are structurally related to ACTH, MSH and β-LPH are modulators of both animal and human behavior [6,7]. Behavioral effects of neuropeptides that are widely studied are those on conditioned avoidance behavior [6,7] and on grooming behavior in the rat [11–13]. In *in vitro* studies in rat brain, interest has focused on the influence of such neuropeptides on various enzymic processes in the plasma membrane. Among the reported neurochemical effects of ACTH-like neuropeptides are those on the production of cAMP [30], and on the phosphorylation of membrane proteins [33–35] and membrane lipids [16,18]. The metabolism of a special class of phospholipids, the polyphosphoinositides, was affected by ACTH, β-endorphin and related peptides [18,19]. In addition, a relationship was shown to exist between polyphosphoinositide metabolism and protein phosphorylation [17].

The present study was designed to investigate whether neuropeptides other than those realted to the ACTH/β-endorphin series may affect brain polyphosphoinositide metabolism. In addition, several neurotransmitters and cyclic nucleotides were tested *in vitro* to ascertain whether the neurochemical action of the neuropeptides resembles those of classical neurotransmitters, and whether the peptide effects might be mediated by cyclic nucleotides.

METHOD

Animals and brain Dissection

Male rats (150 g) of an inbred Wistar strain were used (TNO, Zeist, The Netherlands). After decapitation the brain was rapidly removed. All subsequent operations were performed at 0–4°C. Limbic structures were dissected as described before (hippocampus, septum, basal ganglia, pyriform cortex, diencephalon, mesencephalon) [10].

Subcellular Fractionation

The preparation of the enzyme fraction was performed essentially as described previously [18]. Briefly, the dis-

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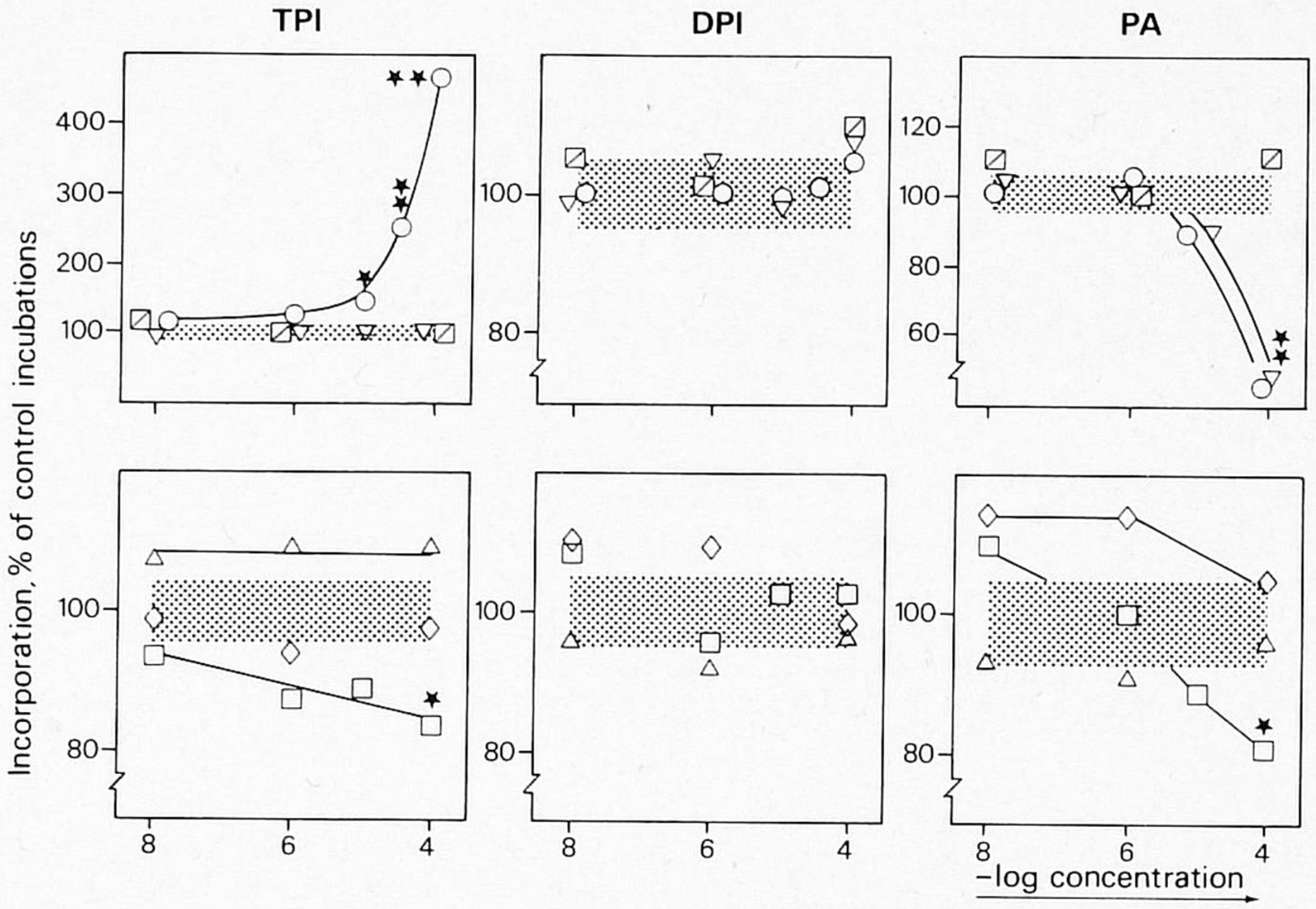

FIG. 1. Peptides/opioids and polyphosphoinositide metabolism. A membrane-cytosol fraction was prepared as described (see METHOD). The membrane samples were preincubated for 5 min at 30°C and the incubation was started by the addition of ATP plus [γ-^{32}P]ATP. Buffer solution plus or minus peptide was added 15 sec before ATP. Incubation time was 10 sec. The results of the incubations with the various substances are expressed as mean percentage of control incubations. The shaded area represents the 100% control value ± standard error of the mean (n=4 for all incubations; SEM ≤ 10%). $^{*}p<0.05$; $^{**}p<0.01$. ○ ACTH; ▽ β-endorphin; ◪ morphine; △ LVP; ◇ angiotensin II, □ substance P.

sected material from one rat (0.3 g) was homogenized in 3 ml homogenization medium (0.32 M sucrose, 1 mM EDTA, 10 mM Tris/HCl, pH 7.4). The crude mitochondrial/synaptosomal pellet (P2) was obtained and subjected to osmotic lysis: the P2 was suspended in 10 vols aqua bidest and stirred for 10 min at 4°C. This suspension was spun for 20 min at 10,000 × g and the supernatant of the lysed P2 (further referred to as membrane-cytosol fraction) was taken as the enzyme fraction.

Phosphorylation Assay

Endogenous phosphorylating activity was assayed as described in detail elsewhere [18]. Briefly, the incubation was performed under the following conditions: 7.5 μM ATP, 3 μCi [γ-^{32}P]-ATP (approx. 3,000 Ci/mmol, Amersham, UK), 50 mM Na-acetate, 10 mM Mg-acetate, pH 6.5 in a final volume of 25 μl (containing approx. 15 μg protein) at 30°C. The membrane samples were preincubated for 5 min and the incubation was started by the addition of ATP. Aliquots of the incubation buffer (5 μl) plus or minus peptides were added 15 sec or 5 min before the ATP (as indicated). The incubations were terminated after 10 sec by the addition of 2 ml ice-cold chloroform/methanol/13 N HCl (200:100:0.75, by vol.; [18]). Protein determination was according to Lowry *et al.* [22].

Lipid Extraction and Thin-Layer Chromatography

After termination of the phosphorylation reaction carrier polyPI (10 μg P) were added and extraction of the labelled phospholipids was performed as described [18]. Briefly, by adding 0.375 ml 0.6 N HCl a biphasic system was obtained. The upper phase was removed and the lower phase was washed 2 times with chloroform/methanol/0.6 N HCl (3:48:47, by vol.). The resulting lower phase containing the polyPI was dried under N_2 and redissolved into chloroform/methanol/water (75:25:2, by vol.). Separation of the labelled phospholipids was performed by one-dimensional High Performance Thin-Layer Chromatography (HPTLC, Merck; layer thickness 25 μm). The plates had been impregnated with K-oxalate and activated for 15 min at 110°C [18]. The solvent was chloroform/acetone/methanol/glacial acetic acid/water (40:15:13:12:8, by vol.). The lipids were visualized with iodine vapour and [^{32}P]-labelled spots were detected by autoradiography on Kodak Royal X-Omat film (10–20 hr). The spots were scraped from the plates and the radioactivity was determined by liquid scintillation counting [18].

Materials

The following synthetic peptides were used: $ACTH_{1-24}$, β-LPH_{61-91} (β-endorphin), substance P, lysine^{8}-vasopressin, angiotensin II. They were obtained from Organon Int. BV (Oss, The Netherlands). Naloxone was obtained from ENDO (New York, USA). Morphine, HCl, acetylcholine and eserine (physostygmine) were from Onderlinge Pharmaceutische Groothandel (Utrecht, The Netherlands). Adrenaline, noradrenaline, serotonin, dopamine, cAMP and cGMP were from Sigma Chemical Co. (St. Louis, USA).

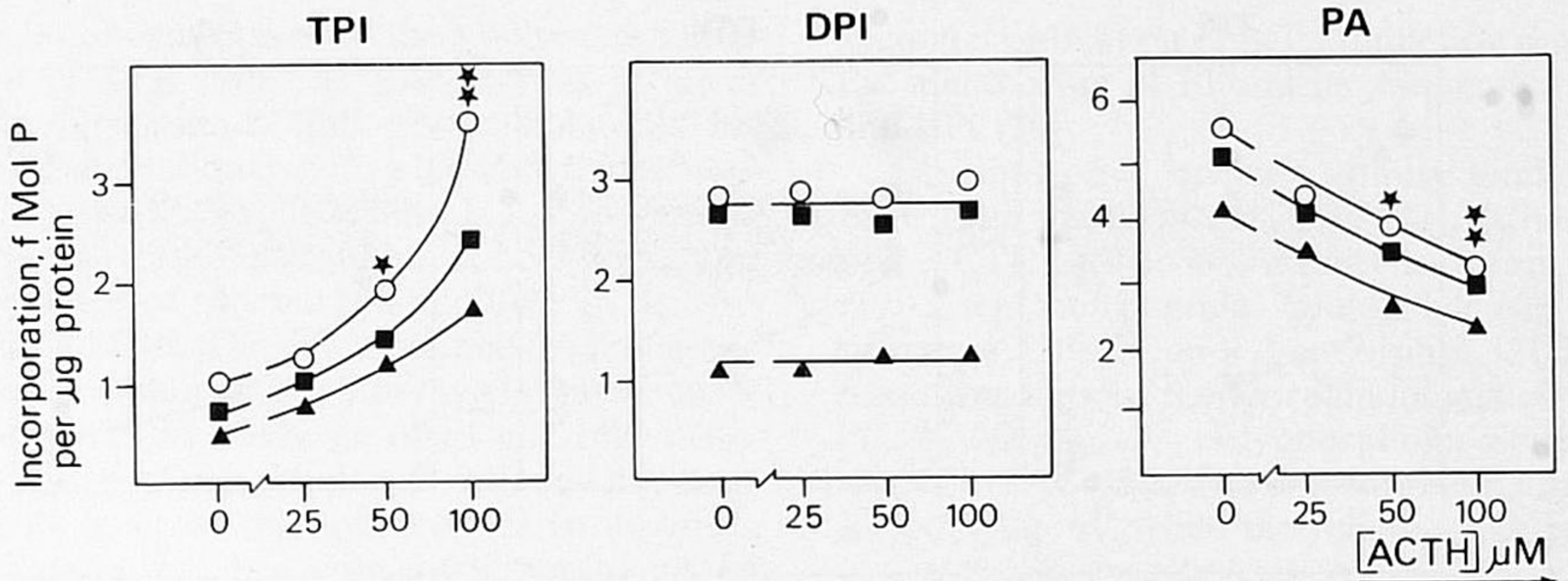

FIG. 2. Effect of preincubation with naloxone and cAMP on ACTH-sensitive polyphosphoinositide metabolism. Incubations were performed as described in the legend to Fig. 1, with the following modifications: the membrane-cytosol fraction was preincubated for 5 min in the presence of naloxone (1 mM; ■) or cAMP (100 μM; ▲), or without any addition (○). $ACTH_{1-24}$ (0, 25, 50, 100 μM) was added 15 sec before the start of the incubation. The results (n=4) are expressed in fmole P per μg protein. $^{*}p<0.05$ in all three conditions; $^{**}p<0.01$ in all three conditions.

Statistics

Differences between groups were tested by one-way analysis of variance followed by the Student's *t*-test [31]. Differences were assigned to be significant for $p<0.05$, two-tailed.

RESULTS

Peptides and Polyphosphoinositide Metabolism

Possible effects of various agents on polyphosphoinositide metabolism were studied in a brain membrane fraction containing cytosolic proteins (see Method). This fraction was incubated for 10 sec in the presence of the agent to be tested and [γ-^{32}P]-ATP. The only phospholipids which were labelled under these conditions were PA, DPI and TPI (data shown in [18]). The reference peptide $ACTH_{1-24}$ stimulated the formation of radiolabelled TPI and inhibited that of PA in a dose-dependent way (Fig. 1). Substance P had an inhibitory influence on both PA and TPI. The peptides [Lys^{8}]-vasopressin and angiotensin II were without an effect at the dose levels tested, whereas β-endorphin had a clear dose-related inhibitory effect on the production of radiolabelled PA.

To investigate whether the influence of β-endorphin and ACTH relates to the opiate-like effects of these peptides, different concentrations of morphine were tested (Fig. 1). No effects of this substance were found. In addition, an attempt was made to counteract the effect of $ACTH_{1-24}$ with the specific opiate-antagonist naloxone: a dose-response curve of the peptide (0, 25, 50 and 100 μM) was made in the presence or absence of naloxone (Fig. 2). The opiate antagonist in high concentrations (1 mM) inhibited TPI and PA formation (30% and −10%, respectively), but the ACTH effects were superimposed on those of the alkaloid, indicating that naloxone does not antagonize these effects of ACTH.

Neurotransmitters and Polyphosphoinositide Metabolism

It was of interest to see if some of the classical neurotransmitters are active under the present conditions in which effects of neuropeptides were obtained. As shown in Fig. 3, dopamine dose-dependently inhibited the formation of radiolabelled TPI, DPI and PA. Serotonin had a similar effect. In contrast, the neurotransmitters noradrenaline, adrenaline and acetylcholine (in the absence or presence of the acetylcholinesterase inhibitor physostygmine) had no significant effect at any concentration tested.

Cyclic Nucleotides and Polyphosphoinositide Metabolism

As shown in Fig. 4, the effects of cAMP on the labelling of TPI showed a biphasic dose-response relationship: a stimulation between 0.5 and 5, and an inhibition above 50 μM. The DPI-labelling was inhibited from 2 μM on, whereas the concentration had to exceed 50 μM to inhibit the formation of radiolabelled PA. No effects of cGMP were found at any concentration tested (Fig. 4).

It was further investigated whether or not ACTH and cAMP affect the metabolism of the polyphosphoinositides at the same level: a dose-response curve of $ACTH_{1-24}$ was made (0, 25, 50, 100 μM) in the presence or absence of the cyclic nucleotide (100 μM). As expected, cAMP lowered the basal level of TPI, DPI and PA labelling (Fig. 2). The ACTH effects on TPI and PA were superimposed on those of the nucleotide, indicating that cAMP did not interfere with the modulatory influence of ACTH. Similarly, the presence of cAMP did not alter the ineffectiveness of ACTH on DPI in this system.

DISCUSSION

The present paper described the influence of various agents on the metabolism of brain polyphosphoinositides *in vitro*. With respect to the effects of the peptides tested, the reference peptide $ACTH_{1-24}$ stimulated the formation of TPI, whereas substance P had an inhibitory effect (Fig. 1). In addition, $ACTH_{1-24}$, substance P and β-endorphin shared a dose-dependent inhibitory effect on PA labelling. Lysine^{8}-vasopressin and angiotensin II did not have any effect on lipid phosphorylation. These effects of substance P are of interest in view of the PI response which is induced by this peptide in rat parotid gland [21].

It would appear from the data (Fig. 1) that certainly not all neuropeptides affect polyphosphoinositide metabolism under these circumstances. Interestingly, the peptides which do affect polyphosphoinositides in the present system, in some way or another can induce excessive grooming behavior in the rat when applied intracerebroventricularly or into

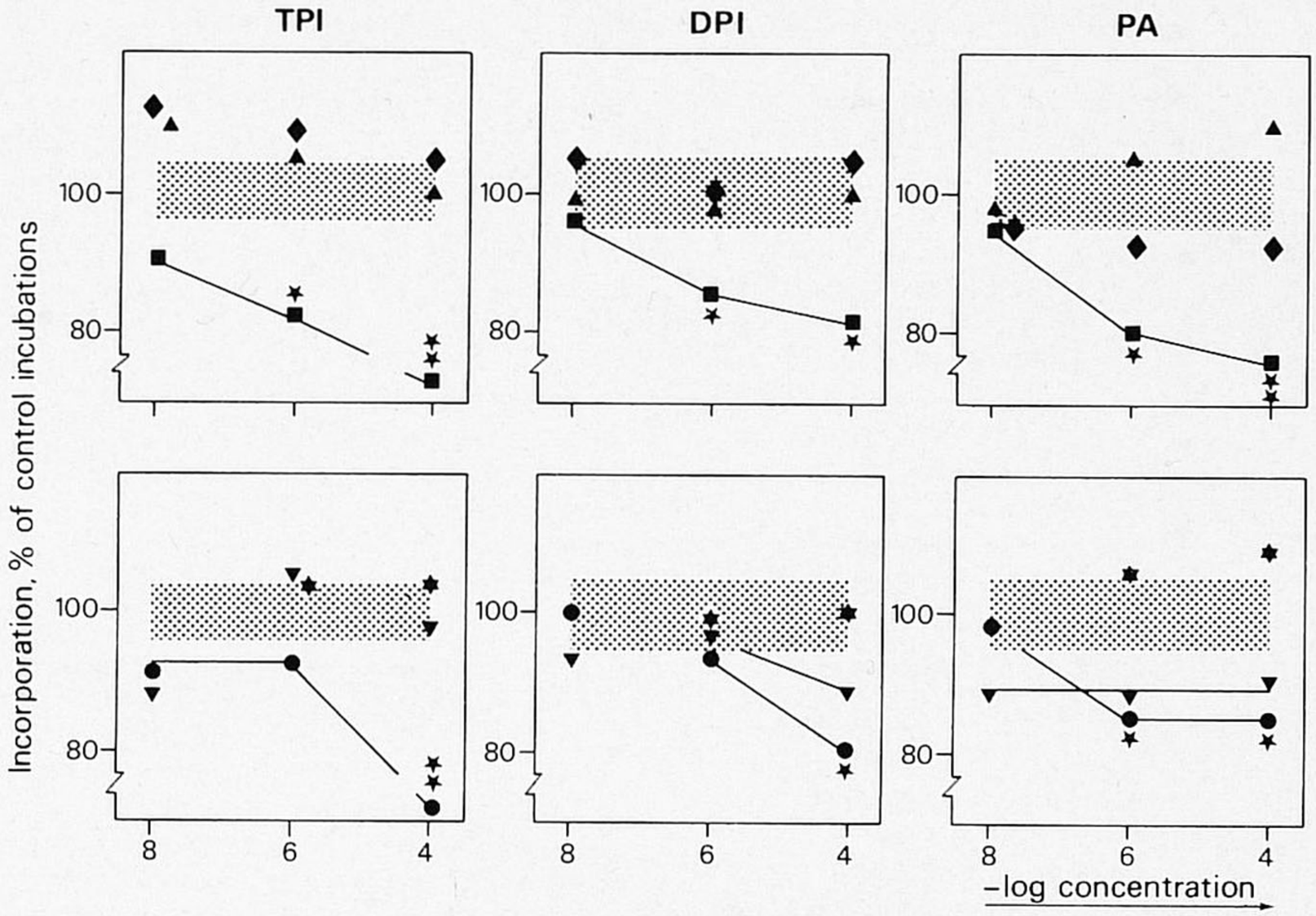

FIG. 3. Neurotransmitters and polyphosphoinositide metabolism. Incubations were performed as described in the legend to Fig. 1. Neurotransmitters were added 15 sec before ATP. The results are explained in the legend to Fig. 1 (n=4). $^{*}p<0.05$; $^{**}p<0.01$. ■ dopamine; ▲ noradrenaline; ◆ adrenaline; ★ acetylcholine + eserine; ● serotonin; ▼ acetylcholine.

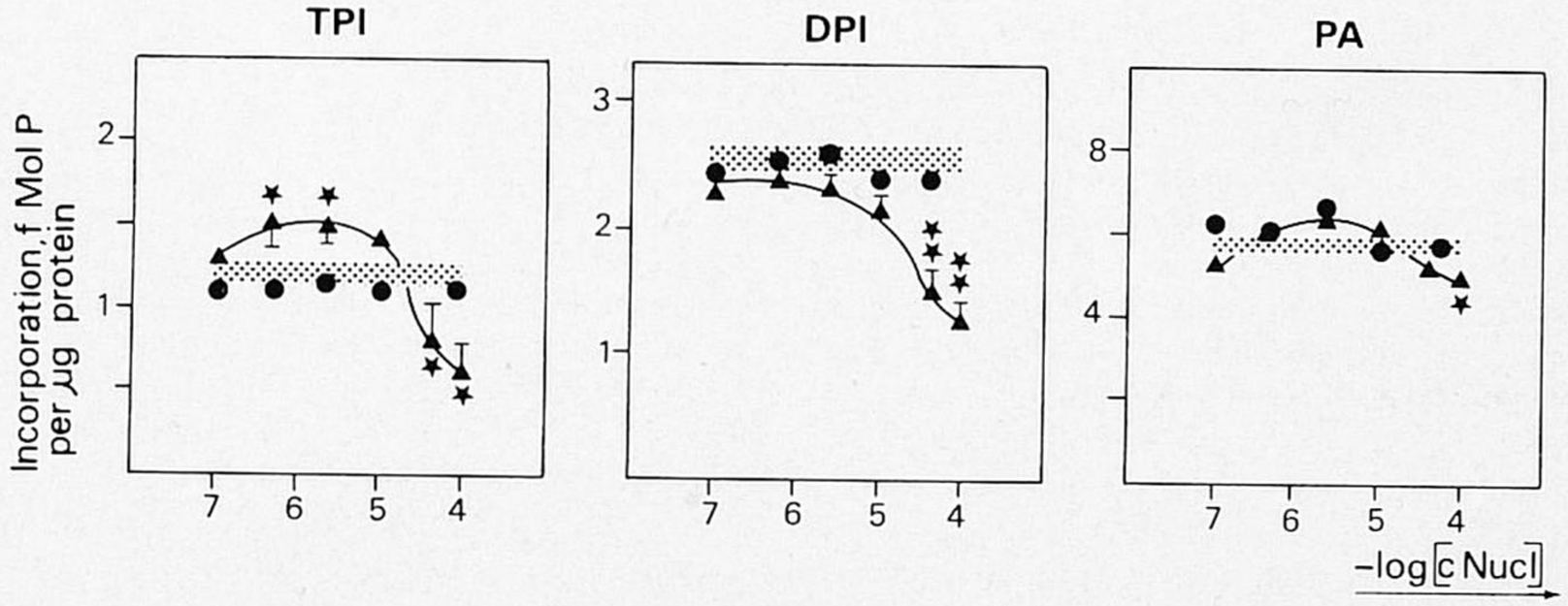

FIG. 4. Effect of cyclic nucleotides on polyphosphoinositide metabolism. Incubations were performed as described in the legend to Fig. 1. Cyclic nucleotides were added 15 sec before ATP. The results are expressed in fmole per µg protein (mean±SEM, n=3, shaded area denotes mean±SEM of the control). $^{*}p<0.05$; $^{**}p<0.01$. ▲ cAMP; ● cGMP.

the substantia nigra [11, 13, 21]. In addition, the structure-activity relationship between ACTH effects on grooming behavior *in vivo* is similar to that obtained on TPI/PA labelling *in vivo* [19]. To what extent such a correlation has a physiological significance is subject to further research (see [20]).

Both dopamine and serotonin dose-dependently inhibited TPI and PA formation, whereas no effects were found with the other transmitters tested (adrenaline, noradrenaline and acetylcholine) (Fig. 3). The effects of PA and serotonin are in the same direction as that of substance P, but differ from those of $ACTH_{1-24}$ and β-endorphin. In view of the difference in pattern of effect after cAMP, the present data make it unlikely that this cyclic nucleotide mediates the effects of dopamine and serotonin in this system (see below).

Up until now little is known of neurotransmitter effects on polyphosphoinositide metabolism in brain membrane preparations in which the integrity of the cell has been lost (e.g., lysed synaptosomes as in the present study or in synaptosomal plasma membrane preparations). In intact cells or synaptosomes effects have been described after muscarinic cholinergic [1,8] and α-adrenergic agonists [2,23] and after dopamine [9,23], histamine [25] and also vasopressin [16]. It is probable that those effects, which are of a long-term nature (10–30 min) are fundamentally different from the short-term effects (seconds) that are obtained in the present, and related studies (see [18,19]).

As has been shown in Fig. 4, cAMP but not cGMP had dose-dependent effects on the formation of the inositol

phospholipids: high concentrations of the nucleotide inhibited the formation of TPI, DPI and PA whereas at lower concentrations, the formation of TPI was stimulated. This biphasic dose-response relationship may indicate that a regulation takes place at two different levels. It is conceivable that the inhibition of TPI labelling at high concentrations of cAMP is a consequence of the marked decrease in the formation of radiolabelled DPI. The data obtained (Fig. 4) are in agreement with those obtained by others: high concentrations of cAMP inhibited the cleavage of PI and the subsequent formation of PA in blood-platelets [5,24], and inhibited the labelling of DPI in renal membranes [4]. In addition, there is some evidence that low concentrations of cAMP might stimulate the formation of TPI [28].

In view of the fact that $ACTH_{1-24}$ and cAMP affect the polyphosphoinositide metabolism differently, it is unlikely that the ACTH effects are mediated by this nucleotide. Besides, the incubation conditions used (10 sec, 7.5 μM ATP, no ATP regenerating system, no cAMP-dependent phosphodiesterase inhibitor present), rule out the possibility that ACTH would act through the production of endogenous cAMP (see also [38]). It is probable that the relationship between polyphosphoinositide metabolism and cAMP metabolism is of another nature: the structure-activity relationship of ACTH-like peptides on the lipids and on adenylate cyclase activity is the same [19,30] see also [20]). Furthermore, anionic phospholipids (notably PI) are essential for basal adenylate cyclase activity and for the restoration of receptor sensitivity [20]. It could thus be that ACTH has a secondary effect on cAMP production due to its influence on the metabolism of PI and its conversion products PA, DPI and TPI [20].

Up until now, no high affinity binding sites for ACTH have been found in the brain [32]. It has only been shown that $ACTH_{1-24}$ has low affinity for brain opiate receptors *in vitro*, as the peptide could displace dihydromorphine, naloxone [26,27] and β-endorphin [3] from their binding sites. In addition, β-endorphin mimicked some of the effects of ACTH on the polyphosphoinositide metabolism (this study, Fig. 1; [19]). The present finding that morphine has no effects (Fig. 1), while the opiate-antagonist naloxone failed to counteract the effects of ACTH (Fig. 2), may indicate that these peptide effects are not mediated by an opiate receptor. Likewise, opiate antagonists (naloxone, naltrexone) failed to counteract the effects of ACTH in its action on brain adenylate cyclase activity [30] and brain membrane phosphorylation (Zwiers, personal communication). However, further research should elaborate on this point as there is evidence from behavioral studies that ACTH may be an endogenous ligand for a morphine receptor that is insensitive to naloxone [15].

In conclusion, the present paper suggests that the influence of ACTH on brain polyphosphoinositide metabolism is rather unique as the other neuropeptides or neurotransmitters tested have different effects or are ineffective. Furthermore, it seems that this influence of ACTH is not mediated by a naloxone-sensitive opiate receptor mechanism or by cAMP.

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