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Phospholipase C activation induced by noradrenaline in rat hippocampal slices is potentiated by GABA-receptor stimulation

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We have studied the effect of γ-aminobutyric acid (GABA) and other GABA-receptor agonists (3-aminopropanesulphonic acid and muscimol) on the noradrenaline-induced stimulation of polyphosphoinositide metabolism in rat hippocampal slices. Formation of water-soluble inositol phosphates, and polyphosphoinositide metabolism were studied in hippocampal slices prelabelled with [^{3}H]*myo*inositol. Noradrenaline induced formation of inositol mono-, bis- and trisphosphate during 10 min incubation in the presence of lithium; activation of phospholipase C by noradrenaline was also reflected by the hydrolysis of polyphosphoinositides and by the increased metabolism of phosphatidylinositol. GABA-receptor agonists were unable to activate *per se* phospholipase C; however, when added together with a low concentration of noradrenaline, they greatly potentiated the noradrenaline-stimulated polyphosphoinositide metabolism. We conclude that GABA-receptor agonists potentiate the effect of noradrenaline on polyphosphoinositide turnover and we discuss the role of this neurotransmitter interaction in the physiology of the hippocampus.

Key words: phosphatidylinositol/noradrenaline/gamma-aminobutyric acid/hippocampal slices/neurotransmitter interaction

Introduction

A number of hormones, neurotransmitters and other ligands, activate polyphosphoinositide hydrolysis upon binding to specific receptors. The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C causes the formation of two putative second messengers: inositol 1,4,5-trisphosphate (IP3), related to intracellular Ca^{2+} mobilization, and 1,2-diacylglycerol (DG), which activates protein kinase C (for reviews see Berridge, 1984; Nishizuka, 1984; Hokin, 1985).

Various neurotransmitters stimulate phospholipase C in the hippocampus; however, despite the conceivable importance of interactions between neurotransmitters acting on this biochemical pathway, only a few reports have appeared on this topic. Recently it has been demonstrated that excitatory amino acid glutamate inhibits carbamylcholine-induced phospholipase C activation, whereas it does not affect polyphosphoinositide metabolism induced by noradrenaline (NA) in hippocampus slices (Baudry *et al.*, 1986).

In this paper we examine the effect of inhibitory γ-aminobutyric acid (GABA) on NA-stimulated polyphosphoinositide metabolism in rat hippocampal slices. In this preparation, NA acts on either α- or β-receptors, respectively inhibiting or enhancing electrical activity (Mueller *et al.*, 1982; Madison and Nicoll, 1986a,1986b). α_1-adrenoreceptors are coupled to phospholipase C activation, and β-adrenoreceptors stimulate the formation of cAMP, which mimics the electrophysiological actions of β-adrenergic stimulation in the hippocampus (Madison and Nicoll, 1986b). GABAergic and noradrenergic transducing pathways are interrelated in the central nervous system; GABA enhances the accumulation of cAMP induced by β-adrenergic stimulation (Karbon *et al.*, 1984). Our results are the first demonstration that GABA potentiates the effect of NA on polyphosphoinositide metabolism, a transmembrane signalling system triggered by activation of adrenoreceptors believed to be responsible for the inhibitory effect of NA on hippocampal electrical activity (Mueller *et al.*, 1982).

Results

Effect of NA on polyphosphoinositide metabolism in rat hippocampal slices

NA (1–100 μM) induces activation of phospholipase C in rat hippocampal slices, presumably via an α_1-adrenoreceptor-mediated mechanism. Activation of phospholipase C by NA has been studied by measuring accumulation of water-soluble inositol phosphates and degradation of polyphosphoinositides (Table I, Figure 1A,B, Figure 2A). Table I shows the accumulation of [^{3}H]inositol phosphates induced by 5 μM NA in slices prelabelled with [^{3}H]*myo*inositol, and incubated with the agonist for 10 min in the presence of 10 mM lithium. NA induces accumulation of inositol monophosphate (IP), inositol bisphosphate (IP2) and IP3. The formation of IP and IP2 is more evident, and this might be due to the relatively long incubation time (see Materials and methods) with the consequent dephosphorylation of IP3 and IP2 by specific phosphatases (Molina y Vedia and Lapetina, 1986). The effect of NA on the metabolism of polyphosphoinositides is shown in Figures 1B and 3B. NA induces a marked degradation of the polyphosphoinositides, whereas, during a 10-min incubation with the agonist, phosphatidylinositol (PI) is resynthesized (Figure 3B). This could be ascribed to activation of the 'PI-cycle' with resynthesis of PI from phosphatidic acid formed through phospholipase C activation (Hokin, 1985).

Effect of GABA on NA-stimulated metabolism of polyphosphoinositides in hippocampal slices

GABA (up 10 mM) does not activate phospholipase C in rat hippocampal slices (Table I and Figure 1B). However, when added together with NA, it significantly potentiates the NA-induced stimulation of polyphosphoinositide turnover. Figure 1A shows the effect of GABA (10 mM) on the formation of inositol phosphates induced by NA (1 μM) in slices prelabelled with [^{3}H]*myo*inositol. It is evident that GABA causes a 90% increase of NA-induced accumulation of inositol phosphates. The effect of GABA on the activation of phospholipase C induced by low concentration of NA is also shown in Figure 1B, which shows that NA induces degradation of [^{3}H]polyphosphoinositides in a dose-dependent manner; GABA (10 mM), which is totally ineffective in the absence of NA, greatly enhances degradation of

Table I. Accumulation of inositol phosphates in rat hippocampal slices prelabelled with [^{3}H]*myo*inositol

Drug additions	Radioactivity in inositol metabolites (c.p.m.)				
	Free inositol + Gro*P*Ins	IP	IP2	IP3	Total inositol phosphates
None (control)	284 070 ± 10 870	764 ± 48	835 ± 80	132 ± 11	1731 ± 91
NA (5 μM)	293 280 ± 12 780	1087 ± 86[a]	1504 ± 96[a]	185 ± 12[a]	2776 ± 149[a]
NA (5 μM) + 3APS (100 μM)	291 516 ± 11 650	1370 ± 93[a,b]	1996 ± 103[a,b]	196 ± 19[a]	3562 ± 169[a,b]
NA (5 μM) + 3APS (100 μM) + BIC (10 μM)	292 980 ± 12 420	1142 ± 119[a]	1615 ± 130[a]	180 ± 11[a]	2937 ± 159[a]
3APS (350 μM)	282 983 ± 11 520	752 ± 56	850 ± 90	120 ± 13	1722 ± 89
GABA (10 mM)	285 235 ± 12 380	770 ± 68	840 ± 89	125 ± 16	1735 ± 100
MUS (10 μM)	283 180 ± 10 940	710 ± 82	861 ± 92	139 ± 20	1710 ± 102
BIC (100 μM)	286 098 ± 11 050	802 ± 80	869 ± 86	145 ± 22	1829 ± 123
CCh (10 μM)	298 910 ± 15 140	2524 ± 120[a]	3180 ± 175[a]	256 ± 26[a]	5960 ± 196[a]
CCh (10 μM) + 3APS (100 μM)	299 500 ± 14 250	2423 ± 110[a]	2996 ± 170[a]	235 ± 25[a]	5654 ± 189[a]

Slices, prelabelled with [^{3}H]*myo*inositol, were incubated for 10 min in the presence of lithium (10 mM), after addition of drugs: noradrenaline (NA), 3-aminopropanesulphonic acid (3APS), bicuculline (BIC), gamma-aminobutyric acid (GABA), muscimol (MUS), carbamylcholine (CCh). [^{3}H]inositol metabolites were extracted, separated, and measured as described. Glycerophosphorylinositol: Gro*P*Ins; inositol monophosphate: IP, inositol bisphosphate: IP2; inositol trisphosphate: IP3.

Results are means ± SEM of four replicate samples in a single experiment, one out of five that gave almost identical results. Statistical significance was assessed by Student's *t*-test.

[a] $P < 0.02$ versus control.

[b] $P < 0.02$ versus NA (5 μM).

polyphosphoinositides by a low concentration of NA. At high concentration of NA (50 μM), this effect of GABA is no longer detectable.

Effect of GABA-receptor agonists on NA-induced phospholipase C activation in hippocampal slices

To ascertain whether the potentiating effect of GABA on phospholipase C activation induced by NA was specific for GABA-receptor stimulation, we investigated the capability of other GABA-receptor agonists to enhance NA-stimulated polyphosphoinositide hydrolysis. Table I shows the effect of 3-aminopropanesulphonic acid (3APS) on NA-stimulated accumulation of water-soluble inositol metabolites. 3APS significantly ($P < 0.02$) enhances the NA-induced formation of inositol phosphates; the effect of 3APS, and that of GABA (not shown, $n = 4$), is antagonized by the $GABA_a$-receptor antagonist bicuculline (BIC), whereas BIC itself does not inhibit NA-induced inositol phosphate formation. This indicates that the $GABA_a$-receptor subtype strongly contributes to the observed effect of GABA and 3APS. Table I also shows that GABA, GABA-receptor agonists and BIC do not affect inositol phosphate formation in the absence of NA. Furthermore, 3APS appears to be ineffective on inositol phosphate formation induced by carbamylcholine (CCh). This might indicate a selective potentiating effect of GABA-receptor agonists on NA-induced polyphosphoinositide metabolism. Figure 2A shows the effect of 3APS (100 μM) on the formation of inositol phosphates elicited by increasing concentration of NA. NA (1−100 μM) induces dose-dependent accumulation of inositol phosphates; 3APS, which is ineffective in the absence of NA, markedly potentiates inositol phosphate accumulation induced by each concentration of NA. On the other hand, Figure 2B shows that 3APS dose-dependently potentiates NA-induced inositol phosphate formation.

Consistently with the previous results, Figure 3A,B shows the effect of 3APS and muscimol on NA-induced polyphosphoinositide metabolism; both GABA-receptor agonists greatly enhance inositol phosphates formation elicited by 1 μM NA (Figure 3A). Figure 3B demonstrates that this effect is actually due to phospholipase C activation with the subsequent triggering of the 'PI-cycle': as shown, 1 μM NA causes degradation of the polyphosphoinositides, and 3APS and muscimol potentiate this event. When receptor stimulation is prolonged, as in these experiments (10 min), PI is then resynthesized (Figure 4B). Again, GABA-receptor agonists potentiate the effect of NA on PI resynthesis which is dependent on phospholipase C activation.

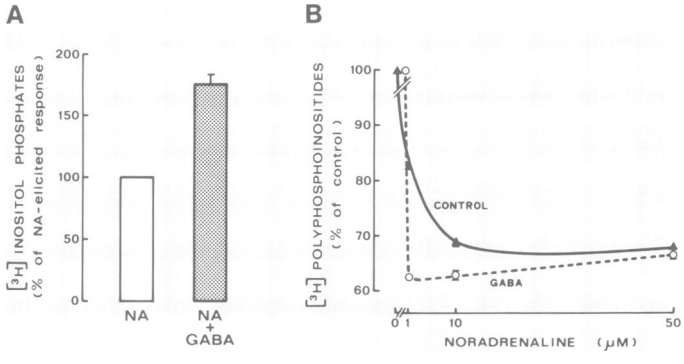

Fig. 1. Effect of GABA on noradrenaline-stimulated metabolism of polyphosphoinositides in rat hippocampal slices. Slices, prelabelled with [^{3}H]*myo*inositol, were stimulated for 10 min by NA in the presence of lithium (10 mM). [^{3}H]inositol phosphates and [^{3}H]polyphosphoinositides were extracted, separated and measured as described. Data are expressed as per cent variations in comparison with basal values. Basal values of a representative experiment were: inositol phosphates, 3689 ± 153 d.p.m.; mean ± SEM of quadruplicate samples. Polyphosphoinositides, 753 ± 67 d.p.m.; mean ± SEM of quadruplicate samples. **(A)** The effect of GABA on the NA-induced accumulation of water-soluble inositol phosphates. GABA, 10 mM; NA, 1 μM (means ± SEM, $n = 5$). **(B)** The effect of GABA (10 mM) on the hydrolysis of polyphosphoinositides induced by increasing concentration of NA. Please note that GABA in the absence of NA does not induce any degradation of polyphosphoinositides. This experiment is representative of 5, which gave very similar results; SEMs of quadruple samples not shown when smaller than symbols.

Discussion

This study demonstrates that GABA-receptor stimulation significantly potentiates the effect of NA on polyphosphoinosit-

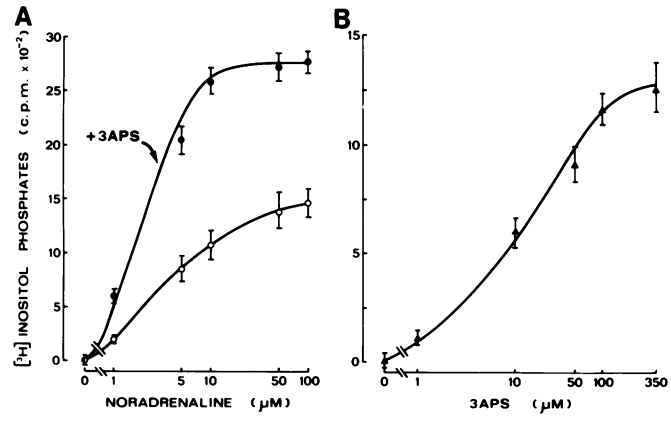

Fig. 2. Effect of 3APS on noradrenaline-induced formation of inositol phosphates in rat hippocampal slices. Assay conditions as in Figure 1. Data are expressed as c.p.m. (means ± SEM, n = 5) after subtraction of control values: unstimulated basal values in **A**, NA-stimulated in **B**. **(A)** The effect of 3APS (100 μM) on the accumulation of [^{3}H]inositol phosphates induced by increasing concentration of NA. **(B)** The effect of increasing concentration of 3APS on the formation of [^{3}H]inositol phosphates elicited by NA (5 μM). Please note different ordinate scale in A and B.

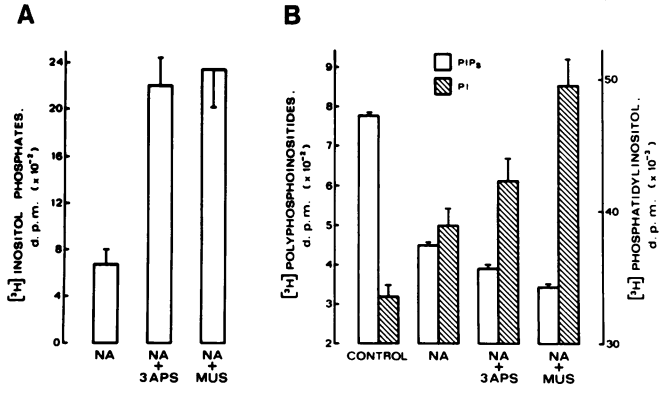

Fig. 3. Effect of 3APS or muscimol (MUS) on NA-stimulated metabolism of polyphosphoinositides in rat hippocampal slices. Assay conditions as in Figure 1. Data are expressed as d.p.m. (means ± SEM, n = 5). **(A)** The effect of 3APS (100 μM) or MUS (1 μM) on inositol phosphate formation induced by NA (1 μM). **(B)** The effect of 3APS (100 μM) or MUS (1 μM) on NA-induced hydrolysis of the polyphosphoinositides (PIPs), and phosphatidylinositol (PI) resynthesis.

ide metabolism in hippocampal slices. Our observation stresses the importance of neurotransmitter interaction on a transmembrane signalling system; it might provide an explanation for the long-lasting enhancement of GABA inhibition of neuronal firing consequent on administration of NA (Yeh *et al.*, 1981).

It has been demonstrated that NA stimulates phospholipase C in rat hippocampal slices by measuring the formation of IP deriving from phosphoinositide hydrolysis (Baudry *et al.*, 1986). The present report adds new information about polyphosphoinositide turnover providing a measurement of IP, IP2 and IP3 formation and the concomitant degradation of polyphosphoinositides and phosphatidylinositol metabolism. Furthermore, our results have been obtained in viable (see Materials and methods) hippocampal slices, i.e. in a preparation that maintains most of its neuronal interconnections, and that is commonly used to investigate the release and the effect of neurotransmitters as well as to study basic mechanisms of memory, e.g. long-term potentiation (Corradetti *et al.*, 1983; Teyler and Discenna, 1984; Kemp *et al.*, 1986; Madison and Nicoll, 1986a).

It is accepted that hydrolysis of PIP2 by phospholipase C is a Ca^{2+}-independent phenomenon, and that IP3 deriving from the hydrolysis is able to mobilize Ca^{2+} from non-mitochondrial endocellular stores (Berridge and Irvine, 1984). However, increasing concentration of intracellular Ca^{2+} induces a variety of changes in inositol phospholipid metabolism. Phospholipase C is further stimulated, and its specificity broadens, so that phosphatidylinositol 4-monophosphate (PIP) and PI are also hydrolysed, with the consequent production of a large amount of DG, which in turn activates protein kinase C (Majerus *et al.*, 1984). Intracellular Ca^{2+} rise is also responsible for direct activation of protein kinase C (Lapetina *et al.*, 1986) and for inhibition of phosphatidylinositol-kinases, or for stimulation of phosphomonoesterases that dephosphorylate PIP2 and PIP with a concomitant increase in PI (Lapetina *et al.*, 1985; Raval and Allan, 1985; Ruggiero *et al.*, 1985).

From our data it appears that GABA-receptor stimulation does not trigger *per se* polyphosphoinositide hydrolysis. In fact, GABA, and other GABA-receptor agonists, are ineffective in stimulating phospholipase C, when added in the absence of NA. This observation rules out the possibility of indirect actions of GABA through release of neurotransmitters which directly activate phospholipase C.

The potentiating effect of GABA-receptor stimulation on NA-induced polyphosphoinositide turnover, can be explained by different hypotheses. One could speculate on the existence of a receptor–receptor interaction, and/or on the interference of the GABA-transducing mechanism with the α-noradrenergic coupling to phospholipase C activation. However, a different explanation can also be proposed. It has been demonstrated that GABA increases Ca^{2+} influx in hippocampal pyramidal cells (Blaxter *et al.*, 1986; Riveros and Orrego, 1986); Ca^{2+} influx could then be responsible for the potentiating effect of GABA-receptor stimulation on NA-induced phospholipase C activation. In this case, then protein kinase C activation would be the main target of this GABA–NA interaction. Protein kinase C would be activated to a greater extent by increasing Ca^{2+} concentration intracellularly, by means of two independent mechanisms: (i) Ca^{2+} favours hydrolysis of PIP and PI by phospholipase C (which is activated by low doses of NA) with the consequent production of high amount of DG (Majerus *et al.*, 1984). (ii) Ca^{2+} can directly activate protein kinase C (Lapetina *et al.*, 1986), and also causes translocation of the kinase from a soluble to a membrane-bound compartment (Wolf *et al.*, 1985).

It has been shown that protein kinase C plays a crucial role in the basic mechanism of long-term memory, that is long-term potentiation (Akers *et al.*, 1986; Berridge, 1986); it has been proposed that high-frequency stimulation of the hippocampus raises intracellular Ca^{2+} concentration with the resulting activation of protein kinase C, and the induction of long-term potentiation (Malenka *et al.*, 1986). Our findings are in agreement with this hypothesis, and show that NA-induced activation of polyphosphoinositide metabolism (with the subsequent Ca^{2+} mobilization and protein kinase C activation) can be greatly enhanced by GABA-receptor stimulation. The GABAergic transducing system would then modulate the α_1-noradrenergic transducing system by potentiating the effect of low concentration of NA. Because NA is known to be involved in long-term potentiation (Stanton and Sarvey, 1985), it is possible that the interaction between GABA and NA on polyphosphoinositide metabolism is related to the biochemical events underlying long-term memory processes.

Materials and methods

GABA, noradrenaline, muscimol, bicuculline methyliodide and carbamylcholine were from Sigma Chemical Co. 3APS was from Aldrich. *myo*[2-^{3}H](N)inositol (10.8 Ci/mmol) was from New England Nuclear. Precoated silica gel G-25 plates (LK6D) were from Whatman. Dowex AG 1 × 8 anion exchange resin (100–200 mesh), was from Bio-Rad. All other reagents were from Carlo Erba, analytical grade.

Preparation and labelling of rat hippocampal slices

Transverse hippocampal slices (350 μM), prepared as previously described (Corradetti *et al.*, 1983), from male Wistar rats (250–300 g) were incubated for 1 h at 37°C under humidified O_2/CO_2 (95:5) in the following Krebs-bicarbonate buffer (mM): NaCl 124, KCl 3.33, Mg_2SO_4 2.42, $CaCl_2$ 3.1, KH_2PO_4 1.25, $NaHCO_3$ 25, D-glucose 10, urea 2, ascorbic acid 2. After further incubation for 90 min with [^{3}H]*myo*inositol (20 μCi/ml) the slices were washed in 50 ml of freshly oxygenated buffer. They were then transferred in tubes (three slices each) with 500 μl of drug-containing medium, gently stirred, in the presence of 10 mM Li^+, by bubbling O_2/CO_2. Some slices were tested electrophysiologically, as previously described (Corradetti *et al.*, 1983), to assess viability. The drugs were allowed to act for 10 min in quadruple samples; then the reaction was stopped by the addition of 1.88 ml of chloroform/methanol/conc. HCl (100/200/2, v/v). Preliminary time-course experiments demonstrated that the earliest time when an effect can be reliably measured is 10 min; this is in agreement with previous studies on inositol phosphate metabolism in the central nervous system (Berridge *et al.*, 1983; Baudry *et al.*, 1986).

Measurement of [^{3}H]inositol phosphates and [^{3}H]inositol lipids in association with stimulation of the slices

Chloroform (0.62 ml) and water (0.62 ml) were then added and, after a brief sonication, the tubes were centrifuged at 800 *g* for 10 min. The upper phase contained the water-soluble inositol phosphates, and portions (1.5 ml) were transferred to test-tubes, and water (2.5 ml) added. The inositol phosphates were then separated on Dowex 1 anion exchange columns as previously described (Lapetina *et al.*, 1985). The following fractions were collected: [^{3}H]inositol and [^{3}H]glycerophosphoryl-inositol (16 ml of 60 mM ammonium formate, 5 mM disodium tetraborate); IP (16 ml of 200 mM ammonium formate, 100 mM formic acid); IP2 (20 ml of 400 mM ammonium formate, 100 mM formic acid); IP3 (12 ml of 1 M ammonium formate, 100 mM formic acid). Control studies using labeled standards had shown that this elution pattern yields 100% recovery of all three inositol phosphates. Radioactivity in portions (8 ml except for [^{3}H]IP3 — 4 ml plus 4 ml of water) of these fractions was determined by scintillation counting using 60% (v/v) Kontrogel (Kontron Analytical). Calculations were performed on each inositol phosphate and on the sum of IP, IP2 and IP3. Data are presented (unless otherwise stated) as means ± SEM of values obtained from different experiments; significance ($P < 0.02$) was assessed by means of Student's *t*-test applied on replicate samples of each experiments. The inositol lipids, located in the lower phase, were dried under nitrogen and resuspended in chloroform (0.05 ml). The inositol phospholipids were then separated on oxalate-impregnated silica gel t.l.c. plates developed in chloroform, methanol, 4 N ammonium hydroxide (45:35:10 v/v). The lipids were visualized by autoradiography and characterized by co-elution with non-labelled standards detected with iodine. The lipids were then scraped and counted by liquid scintillometry.

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