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PROSTAGLANDINS D_2 AND E_2 ARE NOT REGULATORS OF AMINO ACID RELEASE FROM RAT CORTICAL SYNAPTOSOMES

DAVID DRUCE,* JOHN MACDERMOT,† KEITH A. WADDELL, HENRY F. BRADFORD* and IAN A. BLAIR

Department of Clinical Pharmacology, Royal Postgraduate Medical School, Ducane Road, London W12 0HS, and *Department of Biochemistry, Imperial College of Science and Technology, Prince Consort Road, London SW7, U.K.

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Abstract—Rat cerebral cortex synaptosomes synthesise prostaglandins, and analysis by gas chromatography–mass spectrometry revealed that of the prostaglandins quantified PGD_2 (10.9 ng/mg protein) was produced in highest concentration. Treatment with high potassium or veratrine caused release of putative amino acid transmitters, but not of prostaglandins, and prostaglandins D_2 and E_2 were unable to stimulate release of amino acids. The release of putative amino acid transmitters, evoked by high potassium levels, was not inhibited by these prostaglandins. Prostacyclin receptors could not be identified on synaptosomes by radioligand binding techniques.

Prostaglandins (PGs) are synthesised by homogenates or slices of mammalian brain [1–5]. The role of these compounds in the central nervous system remains to be elucidated, although prostaglandin E_2 inhibits noradrenaline release from rat cortical slices [6]. Prostaglandins of the E series also inhibit noradrenergic transmission in the peripheral nervous system [7]. Further investigation of a possible synaptic function of prostaglandins is complicated by contamination of homogenates or slices of cerebral tissue by non-neuronal tissue that synthesises and is sensitive to prostaglandins. These problems were avoided in studies [8–10] of a cloned somatic cell hybrid that was derived from neuronal cells. Prostacyclin (PGI_2) and other prostaglandins activate adenylate cyclase in these cells [8]. The process is receptor mediated [9], and binding of [^{3}H]prostacyclin is regulated by divalent cations [10]. The interaction between the prostaglandin receptor and the adenylate cyclase molecule is dependent on the presence of guanosine 5′-triphosphate, suggesting that the receptor–enzyme coupling is similar to that observed in other hormonal [11] and neurotransmitter [12] systems. In order to extend our studies, rat cortical synaptosomes have been examined. Their ability to synthesise prostaglandins has been investigated. The effect of depolarisation on prostaglandin release, and the effect of prostaglandins on amino acid release has been determined. Finally, radioligand binding techniques have been employed to resolve whether synaptosomes possess a prostacyclin receptor.

MATERIALS AND METHODS

Preparation of synaptosomes. Synaptosomes were prepared by a modification [13] of a previously described method [14]. Whole cerebral cortex was removed rapidly from female adult Sprague–Dawley rats (200–250 g) sacrificed previously by stunning, followed by exsanguination. A 10% (w/v) homogenate was prepared in ice cold sucrose (0.32 M) and centrifuged in a Beckmann type 30 rotor at 1000 *g* for 10 min. The supernatant was retained, and the nuclear pellet (P_1) was resuspended in sucrose (0.32 M) and recentrifuged at 1000 *g* for 10 min. The supernatants were combined and centrifuged for 20 min at 20,000 *g* to yield the P_2 and S_2 fractions. The microsomal supernatant (S_2) was discarded, and the crude mitochondrial pellet (P_2) was resuspended in 45 ml sucrose (0.32 M). An aliquot (15 ml) of this suspension was layered onto a discontinuous sucrose density gradient consisting of 20 ml sucrose (0.8 M) on top of 20 ml 1.2 M sucrose. The sucrose gradients were then centrifuged at 75,000 *g* for 70 min in a Beckmann swing-out rotor, and three fractions were obtained. The synaptosomal enriched fraction P_2B was collected from the 0.8–1.2 M interface. Cold deionised distilled water was added slowly to the synaptosomal fraction, and the suspension was stirred continuously until a sucrose concentration of 0.45 M was reached. The suspension was then centrifuged at 55,000 *g* for 20 min in a Type 30 rotor to give a synaptosomal pellet. The pellet was resuspended in an oxygenated Krebs phosphate medium (NaCl 124 mM, $NaHPO_4$ 20 mM, KCl 5.0 mM, $MgSO_4$ 1.3 mM, $CaCl_2$ 0.75 mM, KH_2PO_4 1.2 mM, glucose 10 mM) pH 7.4, the final concentration was 1–3 mg protein/ml.

Incubation of synaptosomes. A preincubation period of 30 min at 37° allowed the nerve terminals to equilibrate with the medium. For exogenous release studies, the synaptosomes were prelabelled with [U-^{14}C]GABA (224 mCi/mmole, Amersham International, Amersham, U.K.) by including 0.5 μCi/ml of radiolabelled GABA in the incubation

† Author to whom correspondence should be addressed.

medium for a period of 10 min. For endogenous amino acid and prostaglandin studies, synaptosomes were incubated for a period of 10 min in Krebs phosphate medium without additions as control, or stimulated with potassium (55 mM KCl) or veratrine (75 μM) for 10 min. Incubations were terminated by bench centrifugation at 10,000 g for 2 min at room temperature. The pellets were retained for protein determinations [15], and the supernatants were frozen at −20° prior to amino acid or prostaglandin analysis.

Endogenous amino acid analysis. An internal standard of norleucine (10–20 nmoles/ml) was added to the supernatants separated from the incubated synaptosomes. Free amino acids were then extracted from the dried salts and protein by the addition of ice-cold methanol (1 ml). The samples were centrifuged (10,000 *g*, 10 min), and methanol supernatants were decanted off and taken to dryness. The dried extracts were prepared for amino acid analysis by dissolution in 1 ml of 25 mM HCl. Amino acid levels were measured by autoanalyser as described previously [16]. Results were calculated by a small on-line computer (Digico 165) with reference to the standard norleucine signal.

[^{3}H]*Prostacyclin binding assay.* The assay was carried out by a modification of previously described techniques [9]. Synaptosomes were incubated with 20 nM[^{3}H]PGI_2 in 50 mM Tris–HCl buffer pH 8.5 containing 10 mM $MgSO_4$. Incubations were terminated after 15 min at 20°; specific binding was taken as that displaced by 25 μM PGI_2 in parallel incubations.

Prostaglandin analysis. Samples (1 ml) of the supernatants from synaptosome incubations were equilibrated with 25 ng each of 3,3′,4,4′-[$^2H_4$]$PGF_{2\alpha}$, 3,3′,4,4′-[$^2H_4$]PGE_2 and 3,3′,4,4′-[$^2H_4$]-6-oxo-$PGF_{1\alpha}$ and acidified to pH 3 with 0.1 M HCl. Ethyl acetate extraction (2 × 5 ml) was followed by back extraction into 2 M borax buffer (2 ml) pH 8.5. The borax layer was extracted with ethyl acetate, the organic phase discarded, and the aqueous phase adjusted to pH 3 with 0.1 M HCl. The prostaglandins were extracted into ethyl acetate (2 × 2.5 ml), the solvent evaporated under nitrogen at 40°, and the residue transferred in methanol to glass vials. The methanol was removed under nitrogen, methoxamine hydrochloride in dry pyridine (100 μl, 5 mg/ml) was added and the derivatisation allowed to proceed overnight at room temperature. The pyridine was removed *in vacuo*, methanol (0.25 ml) added followed by freshly distilled diazomethane in ether (0.5 ml). The solvent was removed in a stream of nitrogen and the residue redissolved in *N,O*-bis-trimethylsilyl-trifluoroacetamide (25 μl). The samples were heated to 60° for 1 hr to complete the derivatisation, and were then analysed by gas chromatography–mass spectrometry (GC–MS). Standards were carried through the same procedure and standard curves constructed in the range 1–80 ng/ml. Selected ions (*m/z*) monitored were: $PGF_{2\alpha}$, 494 (M-TMSOH); [$^2H_4$]$PGF_{2\alpha}$, 498 (M-TMSOH); PGD_2 and PGE_2, 508 (M-OMe); [$^2H_4$]PGE_2, 512 (M-OMe); TXB_2, 508 (M-OMe-TMSOH); 6-oxo-$PGF_{1\alpha}$, 508 (M-OMe-TMSOH); [$^2H_4$]6-oxo-$PGF_{1\alpha}$, 512 (M-OMe-TMSOH). Standard curves were constructed for $PGF_{2\alpha}$, PGE_2 and 6-oxo-$PGF_{1\alpha}$ with reference to the corresponding heavy isotope internal standard; [$^2H_4$]PGE_2 was employed as the standard for PGD_2 and [$^2H_4$]-6-oxo-$PGF_{1\alpha}$ as the internal standard for TXB_2.

Mass spectrometric conditions. A Finnigan 4000 automated GC–MS system was operated using an electron energy of 25 eV. Separations were achieved by temperature programming a 2 m × 2 mm (i.d.) 3% OV-1 packed column from 200 to 265° at 6°/min with a flow rate of 30 ml/min of helium carrier gas.

Liquid scintillation counting. Radioactive GABA levels in the supernatants were determined using a toluene-PBD based scintillant and a Beckman scintillation spectrometer. [^{3}H]PGI_2 was counted in Insta-Gel (Packard Instrument Co., Caversham, U.K.) on a Packard 3375 liquid scintillation spectrometer.

The following generous gifts were received: PGI_2 from the Wellcome Research Laboratories (Beckenham, U.K.) and PGE_2, PGD_2, $PGF_{2\alpha}$, TXB_2, 6-oxo-$PGF_{1\alpha}$, [$^2H_4$]PGE_2, [$^2H_4$]$PGF_{2\alpha}$, [$^2H_4$]6-oxo-$PGF_{1\alpha}$ from the Upjohn Co. (Kalamazoo, MI).

RESULTS

Suspensions of freshly-prepared synaptosomes in Krebs phosphate medium (pH 7.4) were allowed to equilibrate for 30 min prior to stimulation. Control samples were incubated for a further 10 min, whilst parallel incubations were treated for the same period with selected reagents. The incubations were terminated by centrifugation at 10,000 *g* and the supernatants analysed for prostaglandin content by GC-MS. Under the control incubation conditions, rat cortex synaptosomes synthesised prostaglandins. PGD_2 (10.9 ng/mg protein) was found in the highest concentration (Table 1), while 6-oxo-$PGF_{1\alpha}$ was found in concentrations close to the limit of detection. TXB_2 was not detected. In parallel incubations, in which the synaptosomes were depolarised either with potassium (55 mM KCl) or veratrine (75 μM), there was no measurable increase in the prostaglandin content of the media, whilst differential release of neurotransmitter amino acids did occur, showing the responsiveness of these synaptosomes to the stimulating reagents.

The binding of [11β-^{3}H]PGI_2 to synaptosomes was studied by employing a modification of previously described methods [9]. Experiments were carried out in triplicate and binding of radiolabel was allowed to proceed for 15 min. Specific binding to the synaptosomes represented only 7% of the total binding.

Table 1. Synthesis of prostaglandins by rat cortex synaptosomes

Conditions	$PGF_{2\alpha}$	6-oxo-$PGF_{1\alpha}$	D_2	E_2
Control	5 3 ± 1.6	0 8 ± 0 2	10.9 ± 0 8	6 8 ± 3 3
High K^+	5 2 ± 0 9	0 7 ± 0 2	7 6 ± 1 3	7 6 ± 1 1
Veratrine	4 4 ± 0 3	0.3 ± 0 2	6 8 ± 0 9	4 5 ± 0 5

Results are expressed in ng/mg protein and represent determinations from incubations of synaptosomes ± S.E.M. ($n = 3$).

Table 2. Endogenous release of amino acids by rat cortex synaptosomes

	Asp	Glu	GABA	Gly
Control	118 ± 24	168 ± 23	84 ± 19	371 ± 55
PGD_2 (1 μM)	168 ± 39	238 ± 27	83 ± 10	405 ± 66
PGE (1 μM)	198 ± 71	216 ± 14	96 ± 21	507 ± 121
K^+ (55 mM)	344 ± 38	814 ± 123	466 ± 55	617 ± 84
PGD_2 + K^+ (55 mM)	359 ± 46	991 ± 140	438 ± 59	657 ± 111
PGE_2 + K^+ (55 mM)	357 ± 36	848 ± 102	512 ± 89	711 ± 146

Results are expressed in nmoles/100 mg protein ± S.E.M. ($n = 5$).

A positive control employing NCB-20 neuronal cells in the assay resulted in about 60% specific binding.

The synaptosomes, used in these experiments, were tested for their ability to release physiologically important amino acids both from endogenous and exogenous stores. Incubations were carried out under the same conditions as described for the prostaglandin synthesis. Potassium stimulation evoked release of endogenous aspartate, glutamate and GABA, with only a modest release of other amino acids (Table 2). No measurable release of amino acids occurred following addition of 1 μM PGD_2 or 1 μM PGE_2. These prostaglandins also had no effect on the potassium-dependent release.

When synaptosomes were prelabelled with exogenous [^{14}C]GABA, potassium added to 56 mM caused release of the label (Table 3). No release was evoked with 1 μM PGD_2 or 1 μM PGE_2. Similarly, these prostaglandins had no inhibitory effect on the potassium-dependent release of [^{14}C]GABA.

DISCUSSION

Synaptosomes derived from rat cerebral cortex are able to function as viable biochemical units under appropriate incubation conditions. They function in a similar way to rat cortex slices but are relatively free of non-neuronal prostaglandin sensitive tissue. Synaptosomes are well organised cytoplasmic units which maintain the complexity of the original synapse, and have been used widely as models of central synapses [17]. It has been possible to use this system to detect the release of putative neurotransmitters, and also to investigate the factors that regulate their release.

Analysis of the supernatants from synaptosomal incubations in the present experiments has revealed that cortical synaptosomes synthesise prostaglandins. By employing a stable isotope dilution analysis based on GC–MS, PGD_2, PGE_2, $PGF_{2\alpha}$ and 6-oxo-$PGF_{1\alpha}$ were identified in the supernatants. Quantification by means of the appropriate heavy isotope internal standard showed that PGD_2 was the most abundant prostaglandin formed, with 6-oxo-$PGF_{1\alpha}$ being formed only in amounts close to the limit of detection. These findings agree with earlier studies [3] which demonstrated that PGD_2 is the most abundant prostaglandin formed from homogenates of rat cortex.

Table 3. Release of exogenous GABA by rat cortex synaptosomes

Sample	5 mM K^+	55 mM K^+
Control	1.63 ± 0.22	4.03 ± 0.79
Control + PGD_2	1.77 ± 0.21	4.43 ± 0.90
Control + PGE_2	1.60 ± 0.25	4.37 ± 0.86

Results are expressed as dpm/100 mg protein ± S.E.M. ($n = 8$–11).

Stimulation of synaptosomes with either potassium or veratrine caused no detectable further release of prostaglandins beyond control levels. Thus, depolarisation in this system is not likely to be a functionally important mediator of prostaglandin release.

Previous studies have demonstrated that prostaglandins mediate activation of adenylate cyclase in neuronal cells [8]. This biological activity was shown to be dependent on the occupation of a specific prostacyclin receptor which was characterised by radioligand binding techniques [9]. The present study employing the rat cortical synaptosomes, has demonstrated no significant binding of [^{3}H]PGI_2. The possibility that prostaglandin receptors are located on synaptosomes cannot be totally excluded, but they would have to be present at very low concentrations.

Although depolarisation of synaptosomes with high potassium concentrations caused release of aspartate, glycine and GABA, with a more modest release of other amino acids, PGD_2 and PGE_2 caused no increased release. Prostaglandins D_2 and E_2 also produced no inhibition of the potassium-evoked amino acid release. Experiments in which exogenous radiolabelled GABA was taken up into the synaptosomes demonstrated that although potassium partially released preloaded [^{14}C]GABA, neither PGE_2 nor PGD_2 was able to. These results contrast with the demonstration that PGE_2 is a potent inhibitor of potassium-evoked noradrenaline release from slices of rat cerebral cortex [6].

In conclusion, cerebral cortical synaptosomes synthesise prostaglandins. They release putative amino acid transmitters on depolarisation but not when treated with PGE_2 or PGD_2. These prostaglandins have no inhibitory effect on potassium-evoked amino acid release. Finally, prostaglandins are not released by depolarisation with potassium or veratrine.

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