Br. J. Pharmac. (1969), 37, 698–704.

The release of prostaglandin E_1 from the rat phrenic nerve-diaphragm preparation

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1. The release of prostaglandin E_1 from the rat phrenic nerve-diaphragm preparation during tetanic contraction of the muscle in response to nerve stimulation is reported. Tentative identification of the prostaglandin depended on solvent extraction column and thin-layer chromatography and parallel biological assay.

2. Polar lipid substances were released from the preparation in the absence of nerve stimulation by (+)-tubocurarine and noradrenaline.

3. The output on nerve stimulation was not abolished by (+)-tubocurarine, hemicholinium, bretylium or phenoxybenzamine added to the bath.

4. The possible origin of these prostaglandins is discussed.

Ramwell, Shaw & Kucharski (1965) reported that prostaglandin E_1 (PGE_1) is released from the rat isolated diaphragm on stimulation of the phrenic nerve. This release was not affected by either (+)-tubocurarine or physostigmine. Furthermore, noradrenaline added to the organ bath also caused the release of a prostaglandin. These workers suggested that the release on nerve stimulation may result from stimulation of adrenergic fibres in the phrenic nerve.

In this paper more detailed evidence is presented that the prostaglandin released from the diaphragm on nerve stimulation is mainly PGE_1 and that not only (+)-tubocurarine but also hemicholinium, bretylium and phenoxybenzamine fail to block the prostaglandin output in response to nerve stimulation. Furthermore, (+)-tubocurarine and phenoxybenzamine themselves cause prostaglandin release in the absence of nerve stimulation.

Methods

Rat phrenic nerve-diaphragm preparation

Hemidiaphragms from rats (150–200 g) of either sex were suspended in a 40 ml. bath containing Krebs solution gassed with 95% oxygen and 5% carbon dioxide at 37·5° C. The composition of the Krebs solution was as follows (g/l.): NaCl, 6·92 ;

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KCl, 0·354; $CaCl_2$, 0·282; KH_2PO_4, 0·162; $MgSO_4 7H_2O$, 0·294; $NaHCO_3$, 2·1; glucose, 2·0.

In every experiment two identical baths were used, and either one or two hemidiaphragms were suspended in each bath. Equal periods of incubation were used for control and treated tissues.

The phrenic nerve was stimulated with supramaximal square wave pulses of 0·05 msec duration at a frequency of 25/sec.

In addition to experiments involving nerve stimulation, unstimulated diaphragms were incubated in Krebs solution before and after the addition of various drugs. The bathing fluid was collected at the end of the control period and after a similar period following the addition of the drug.

Extraction procedure

The bath fluid was adjusted to pH 3 with hydrochloric acid and partitioned twice with an equal volume of ethyl acetate. The subsequent extraction procedure was that of Davies, Horton & Withrington (1968).

Biological assay

Initially prostaglandins were estimated on the uterine horn from rats ovariectomized at least 14 days previously (Hawkins, Jessup & Ramwell, 1968). In later experiments the rat fundus strip (Vane, 1957) was used. Contractions were recorded with an Ether force displacement transducer coupled to a Servoscribe pen recorder.

Results

The results are summarized in Table 1.

Output of prostaglandins from the rat isolated diaphragm

In a preliminary experiment matched pairs of rat hemidiaphragms were suspended in two baths. One muscle was stimulated by its nerve and its tetanic contraction was recorded; the other control hemidiaphragm was not stimulated. The fluid

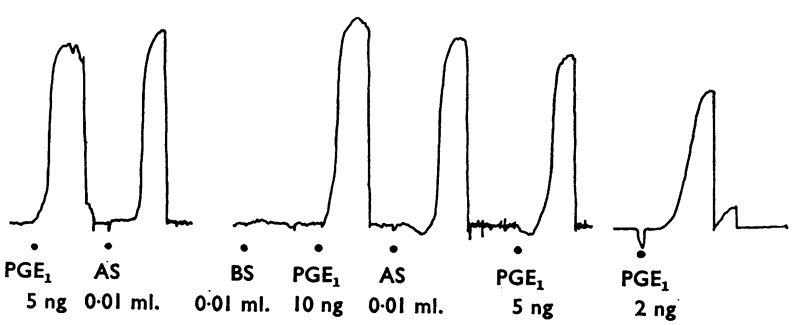

FIG. 1. Rat isolated uterus. Isometric contractions in response to PGE_1 and to 0·01 ml. samples (from a total volume of 1·0 ml.) of the extracted bath fluid from the rat phrenic nerve-diaphragm preparation collected before (BS) and after (AS) electrical stimulation of the phrenic nerve.

from the two organ baths was extracted and assayed separately for prostaglandin content. In this experiment no prostaglandin ($\ll$ 10 ng PGE_1) could be detected in the fluid from the unstimulated diaphragm, whereas that from the stimulated muscle contained the equivalent of about 10 ng PGE_1 (Table 1).

In further experiments larger numbers of diaphragms and longer periods of stimulation were used. In one, the total prostaglandin output was 135 ng PGE_1 equivalent, compared with 15 ng in the control; in another the values were, respectively, 1,500 ng and 25 ng PGE_1 (Table 1). After an experiment using the full extraction procedure, the values were 700 ng and <200 ng, respectively (experiment of Fig. 1).

Evidence that the substance released is prostaglandin E_1

By pooling samples from diaphragms stimulated in the absence of drugs, sufficient material was collected to attempt a tentative identification. The bath fluid was subjected to a series of solvent partitions, which separate polar acidic lipids including the prostaglandins from other substances. At the end of such a solvent extraction procedure, the extract contained most of the original biological activity as tested on the rat fundus. The extract was then chromatographed on a silicic acid column.

TABLE 1. *Amount of prostaglandin-like activity released from the rat diaphragm preparation in response to various treatments, expressed in terms of PGE_1 equivalent*

Expt. No.	No. of hemi-diaphragms for treatment	Control	PGE_1 equivalent (ng)	Test	PGE_1 equivalent (ng)
1	1	Unstimulated	$\ll 10$	Nerve stimulation at 25/sec for 15 min	10
2	4	Unstimulated	< 15	Nerve stimulation at 25/sec for 20 min	135
3	14	Unstimulated	< 25	Nerve stimulation at 25/sec for 20 min	1,500
4*	14	Unstimulated	< 200	Nerve stimulation at 25/sec for 20 min	700
5	4	Control nerve stimulation	143	Nerve stimulation in presence of bretylium 12·5 μg/ml.	258
6	4	Control nerve stimulation	68	Nerve stimulation in presence of bretylium 15 μg/ml.	88
7	4	Control nerve stimulation	70	Nerve stimulation in presence of bretylium 15 μg/ml.	16
8*	16	Control nerve stimulation	500	Nerve stimulation in presence of bretylium 15 μg/ml.	540
9	4	Control nerve stimulation	131	Nerve stimulation in presence of (+)-tubocurarine 50 μg/ml.	440
10	4	Unstimulated	125	Unstimulated in presence of (+)-tubocurarine 50 μg/ml.	1,000
11*	4	Unstimulated	79	Unstimulated in presence of (+)-tubocurarine 50 μg/ml.	350
12	4	Nerve stimulation	165	Nerve stimulation in presence of hemicholinium 100 μg/ml.	168
13	4	Unstimulated	< 250	Unstimulated in presence of noradrenaline 10 μg/ml.	500
14	4	Unstimulated	63	Unstimulated in presence of noradrenaline 10 μg/ml.	306
15*	4	Nerve stimulation	113	Nerve stimulation in presence of phenoxybenzamine 10 μg/ml.	500
16*	4	Unstimulated	63	Unstimulated in presence of phenoxybenzamine 10 μg/ml.	75

*Full extraction procedure used (Davies, Horton & Withrington, 1968). In all the other experiments samples were assayed after extraction of acidified bath fluid with ethyl acetate.

Assays in experiments 1–4 were done on the non-oestrous rat uterus. All the other assays were performed on the rat fundus strip.

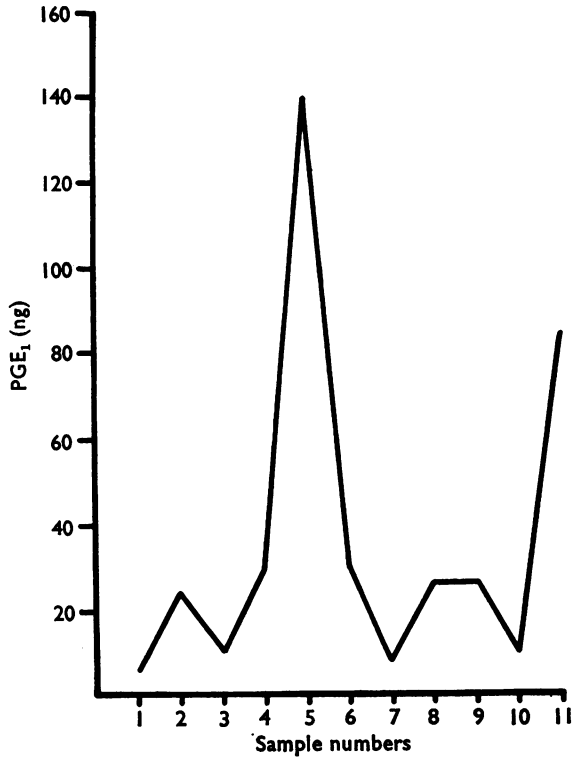

FIG. 2. Silicic acid column chromatogram of material extracted from bath fluid collected after phrenic nerve stimulation. Ordinate, biological activity in terms of PGE_1 assayed on the rat fundal strip. Abscissa, fraction numbers. Fractions were eluted with increasing concentrations of ethyl acetate in benzene: 1 and 2, 30% ; 3–7, 40% ; 8 and 9, 80% and 10, 100%. Fraction 11 was eluted with methanol.

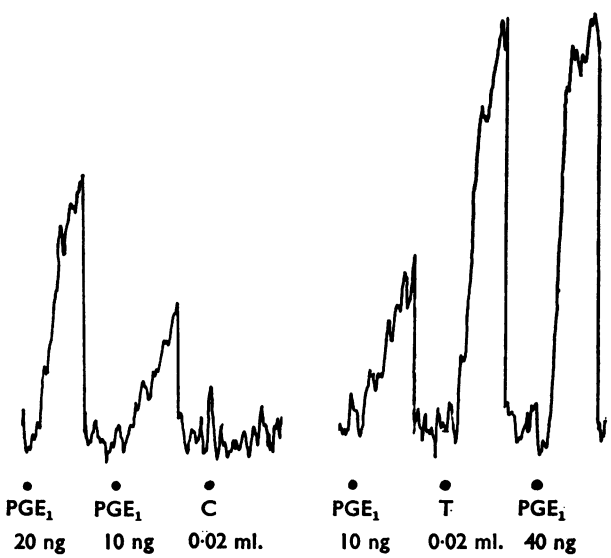

FIG. 3. Rat fundal strip. Isometric contractions in response to PGE_1 and extracted bath fluid from rat phrenic nerve diaphragm preparation collected in the absence of nerve stimulation and in the absence (C) and presence (T) of (+)-tubocurarine (50 μg/ml.).

Figure 2 illustrates the separation of two peaks of biological activity. The first of these corresponds to the elution time for prostaglandins of the E series. This material (fractions 4–6) was pooled and, using Green & Samuelsson's (1964) A II system, chromatographed on a thin-layer plate of silica gel impregnated with silver nitrate: 90% of the biologically active material on the plate was eluted from the zone corresponding to the R_F value of PGE_1 (0·75) and the remaining 10% was located in the PGE_2 zone. On parallel biological assay, using the rat fundal strip and rat uterus estimates of the amounts of PGE_1 present agreed when assayed in terms of pure PGE_1.

Effects of (+)-tubocurarine on output

When the phrenic nerve was stimulated in the presence of (+)-tubocurarine (50 μg/ml.), tetanic contraction of the muscle did not occur but prostaglandin release was not abolished. Indeed there was a greater output of polar lipid on nerve stimulation in the presence of (+)-tubocurarine (Table 1). This suggested that (+)-tubocurarine itself might release a polar lipid. When paired unstimulated preparations were studied, the output of polar lipid in the presence of (+)-tubocurarine was 5–8 fold greater than in the control (Fig. 3).

The nature of the polar lipid released from the unstimulated rat phrenic nerve-diaphragm preparation by the addition of (+)-tubocurarine was not conclusively established. On solvent partition (Davies *et al.*, 1968) it behaved like a polar acidic lipid, but on silicic acid chromatography its elution time was slower than most of PGE_1. A large amount (90%) of biological activity was eluted from the silicic acid with 100% methanol. No attempt was made to identify the active principles.

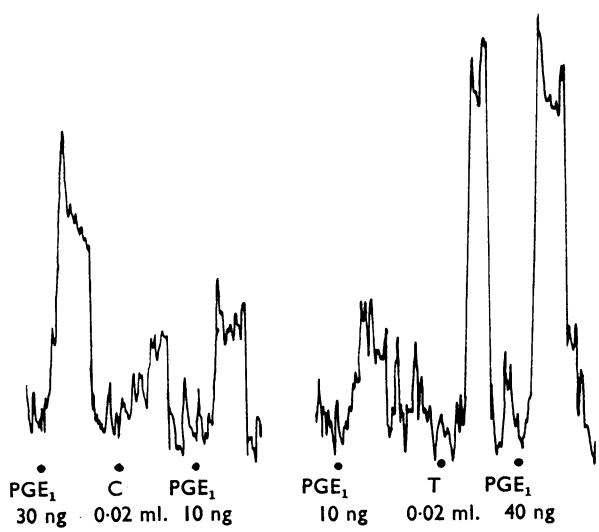

FIG. 4. Rat fundal strip. Isometric contractions in response to PGE_1 and extracted bath fluid from the rat phrenic nerve-diaphragm preparation collected in the absence (C) and presence (T) of noradrenaline (10 μg/ml.).

Effect of noradrenaline on prostaglandin output

On addition of noradrenaline 10 μg/ml. to the bath there was an increase in prostaglandin-like activity compared with fluid taken from the control diaphragms (Fig. 4). This material behaved like a prostaglandin on solvent partition, but its precise identity was not established owing to the relatively small quantities available.

Effects of bretylium tosylate on prostaglandin output in response to phrenic nerve stimulation

If prostaglandin release from the phrenic nerve-diaphragm preparation results from the stimulation of adrenergic fibres, then blockade of these fibres with bretylium tosylate might also block the prostaglandin release. Paired hemidiaphragms were stimulated via their nerves. Bretylium tosylate (12·5 or 15 μg/ml.) was present in one bath. This concentration of bretylium did not modify the muscle contraction in response to nerve stimulation. In two out of three experiments in which the material collected from four hemidiaphragms was pooled there was no reduction in prostaglandin output in response to nerve stimulation. In a fourth experiment (No. 8) with sixteen pairs of hemidiaphragms there was also no reduction in output.

Effect of hemicholinium on prostaglandin output in response to nerve stimulation

If prostaglandin release from the phrenic nerve-diaphragm preparation results from stimulation of cholinergic fibres, then blockade of these fibres with hemicholinium should also block prostaglandin release. In one experiment hemicholinium (100 μg/ml.) was added to the bath. Contraction of the diaphragm in response to phrenic nerve stimulation was completely abolished, but the output of prostaglandin was equal to that from the control diaphragm, which had contracted.

Effect of phenoxybenzamine on prostaglandin output in response to nerve stimulation

The prostaglandin output from four hemidiaphragms in response to nerve stimulation was not abolished in the presence of phenoxybenzamine (10 μg/ml.); indeed the output was greater. Using four unstimulated hemidiaphragms no evidence was obtained that phenoxybenzamine alone causes release of prostaglandin.

Discussion

The results reported here support the findings of Ramwell, Shaw & Kurcharski (1965) that PGE_1 is released from the rat phrenic nerve-diaphragm preparation on nerve stimulation, that this output is not abolished by (+)-tubocurarine and that release also occurs in the presence of noradrenaline. The evidence presented here that the prostaglandin is principally E_1 with about 10% PGE_2 is more detailed than that of Ramwell *et al.*, but it is still open to many objections, such as those discussed at length by Horton & Main (1967). Identification by means of chromatographic and biological data is at best tentative.

It is of importance to consider the origin of these prostaglandins. Since prostaglandins are also released on nerve stimulation when contractions of the diaphragm have been blocked by curare or hemicholinium, the release does not result from

muscle contractions. The release of prostaglandin-like substances from this preparation by (+)-tubocurarine in the absence of nerve stimulation reduces the value of this drug as a tool. It is quite conceivable that the output of prostaglandin in response to nerve stimulation is, in fact, blocked by (+)-tubocurarine, but that this block is overshadowed by the prostaglandin released by (+)-tubocurarine itself. However, hemicholinium abolished the muscle contraction, presumably by blockade of cholinergic neurones, and did not prevent prostaglandin release ; moreover, there was no evidence that this drug itself releases prostaglandin.

The failure of both bretylium and phenoxybenzamine to block prostaglandin output on nerve stimulation does not support the hypothesis that this output is secondary to the release of noradrenaline from stimulated adrenergic fibres. Since the target organ of these hypothetical adrenergic fibres is not known, there was no objective evidence that the pathway was in fact blocked by phenoxybenzamine. On the other hand, the concentrations used of both bretylium and phenoxybenzamine were in excess of those needed to produce effective blocks in other isolated preparations.

It is apparent that the release of prostaglandins from tissues is a widespread phenomenon and that a multiplicity of forms of stimulation will cause such release. There have been suggestions that the formation and release of prostaglandins may occur in many, perhaps all, tissues when cell membranes are activated. In the phrenic nerve-diaphragm preparation the output may well be derived from both pre- and post-synaptic sites, and the possibility of a contribution from tissue, other than muscle or nerve, for example, adipose tissue and mast cells, cannot be excluded.

In conclusion, the present experiments do not support the suggestion that prostaglandin release from the phrenic nerve-diaphragm preparation results solely from the adrenergic nerve stimulation. The observation that drugs such as (+)-tubocurarine can cause prostaglandin release underlines the need for caution in the use of drugs as specific blocking agents. The detection of more polar lipids with biological activity suggests that PGE_1 is only one of a group of substances released under the conditions of our experiments.

I thank Professor E. W. Horton for his inspiration and help, Messrs. Upjohn Ltd., U.S.A., for a gift of PGE_1, and Mr. R. Barclay for able technical assistance.

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(*Received May* 12, 1969)