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The role of prostacyclin in modulating cholinergic neurotransmission in guinea-pig ileum

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1 The mechanism of action of prostacyclin (PGI_2) on isolated segments of guinea-pig terminal ileum was studied by recording the changes in isometric tension.

2 In these preparations PGI_2 (1 nM–1 μM) caused a concentration-dependent increase in muscle tension. This effect was rapid and short-lasting.

3 PGI_2-induced contractions were inhibited by atropine and potentiated by physostigmine.

4 Hemicholinium-3 reduced the response to PGI_2 and the inhibition was quantitatively comparable at any PGI_2 concentration tested.

5 Tetrodotoxin as well as low temperature (20°C) abolished and β-bungarotoxin reduced the effect of PGI_2.

6 Hexamethonium decreased the response to submaximal, but not to maximal PGI_2 concentrations.

7 PGI_2 potentiated the twitch response of the ileum to electrical stimulation.

8 In the presence of tetrodotoxin, PGI_2 did not alter the effect of a sub-maximal concentration of acetylcholine (ACh).

9 The present results give indirect evidence for the ability of PGI_2 to facilitate ACh release from intramural nerves possibly by increasing the excitability of cholinergic cell bodies.

Introduction

Several studies have demonstrated that prostaglandins of the E series (PGE) are released from the guinea-pig ileum under different experimental conditions (Botting & Salzmann, 1974; Botting, 1977; Kadlec, Masek & Seferna, 1978; Yagàsaki, Takai & Yanagiya, 1980) and contract the longitudinal smooth muscle of this intestinal segment (Bennett, Eley & Scholes, 1968). Even though the attempts to clarify the mechanism of action PGE_1 and PGE_2 in the ileum have led to conflicting results, there is now good evidence of the ability of these prostaglandins to increase acetylcholine (ACh) output from nerve endings (Kadlec, Masek & Seferna, 1974; Hall, O'Neill & Sheehan, 1975; Kadlec *et al.*, 1978; Yagasaki, Takai & Yanagiya, 1981).

Like PGEs, prostacyclin (PGI_2) is synthesized by human and animal intestinal tract (LeDuc & Needleman, 1979; Bennett Hensby, Sanger & Stamford, 1981; Whittle, 1981) and contracts guinea-pig ileum (Moncada, Gryglewski, Bunting & Vane, 1976; Sirois, Borgeat & Jeanson, 1981).

The studies presented in this paper were undertaken in order to elucidate the mechanism of PGI_2-induced contractions in the terminal ileum and the possible role of PGI_2 in the modulation of cholinergic transmission.

Methods

Guinea-pigs of either sex (300–500 g body wt.) were killed by a blow to the head followed by exsanguination. The terminal portion (20–25 cm) of the ileum was immediately excised, cleaned and kept in a Tyrode solution of the following composition (mM): NaCl 136, KCl 2.7, $CaCl_2$ 1.4, $MgCl_2$ 0.49, NaH_2PO_4 0.32, $NaHCO_3$ 12 and glucose 5. After the 8–10 cm nearest to the ileo-caecal junction had been discarded, 2.0–2.5 cm segments were mounted vertically in an organ bath (10 ml) under a tension of 1 g and changes in tension were recorded by means of a Basile isometric transducer-Recorder Gemini 7070 System. The bath fluid was aerated Tyrode solution maintained at 37°C. Drugs were diluted in saline (0.9% w/v NaCl solution) or in absolute ethanol and added to the bath fluid in volumes of 0.01 ml. The

preparations were allowed to equilibrate for 40 min, with regular changes of the Tyrode solution every 10 min. After this period each preparation was challenged with ACh (0.2 μM; 0.4 μM; 1 μM). Exposure to the first concentration for 1 min was followed by 3 consecutive washes (at 2 min intervals) and 10 min rest, in order to allow the tracing to come back to the base line before the next challenge with ACh. Maximum contraction was elicited by a concentration of 0.4 μM ACh. Stock solutions of PGI_2 (2 mM) in absolute ethanol were stored at −35°C and diluted with ethanol immediately before use.

Concentration-response curves to acetylcholine and prostacyclin

The preparations were challenged with the lowest effective concentration of ACh or PGI_2 for 1 min. The action was terminated by washing with Tyrode solution. During the next 20 min the preparations were washed again twice, before a higher concentration of ACh or PGI_2 was added. The procedure was repeated with increasing concentrations, until there was no further increase in the contraction. Either the two drugs were tested consecutively on the same preparation or two adjacent segments of the ileum were used for the treatment with ACh or PGI_2. No difference was observed between the results obtained with the two procedures.

Effect of atropine

The effect of various atropine concentrations (1–30 nM) which were predetermined to be in the range required to inhibit ACh-induced contractions, was tested in separate adjacent ileum segments.

Each preparation was first challenged with PGI_2 (20 μM) for 1 min. After washing and waiting 20 min for re-equilibration, atropine was added to the bathing fluid and allowed to act for 3 min before the addition of PGI_2.

Influence of physostigmine, hemicholinium-3, tetrodotoxin and β-bungarotoxin

The preparations were challenged with 20 μM PGI_2 for 1 min and then washed. After 20 min, during which 2 more washes were performed, physostigmine (50 nM), hemicholinium-3 (20 μM), tetrodotoxin (10 nM) or β-bungarotoxin (5 μg ml^{-1}) was added to the Tyrode solution in the bath. The drugs were allowed to act for 10, 30, 3 or 60 min respectively before the preparations were challenged again with PGI_2.

Influence of hexamethonium

Adjacent segments of the same ileum were used in these experiments. One was bathed with normal Tyrode solution, the other with the same solution containing 0.1 mM hexamethonium bromide. Both preparations were then challenged in parallel with increasing concentrations of PGI_2 as described above.

Electrical nerve stimulation

Ileum segments were placed between two parallel platinum electrodes connected to a Grass stimulator mod. S4KR and stimulated with rectangular pulses of 1 ms duration at a frequency of 0.1 Hz and of sufficient strength (18–30 V) to produce a maximal or submaximal response to a single shock.

Evaluation of data

The contractions induced by PGI_2 and ACh were measured and expressed as a percentage of the maximum contraction elicited by ACh (0.4 μM) in the same preparation. In the experiments in which the effect of atropine was tested, PGI_2 contractions measured in the presence of the antagonist were referred to the corresponding contraction elicited by PGI_2 alone (100%).

The significance of the difference between the data groups was evaluated by using Student's *t* test.

Drugs

Acetylcholine bromide, hexamethonium bromide, β-bungarotoxin (snake toxin from *Bungarus multicinctus*) were purchased from Sigma Chemical Co., U.S.A.; tetrodotoxin (Tarichatoxin) and physostigmine (eserine salycilate salt) were from Boehringer, Germany; atropine sulphate from Merck, Germany; hemicholinium from Serva, Ger-

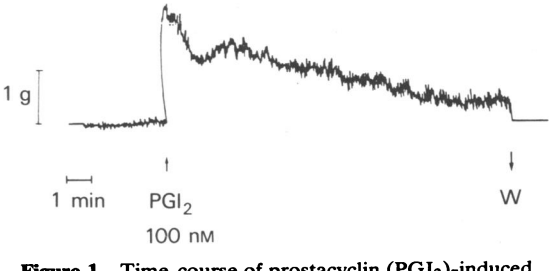

Figure 1 Time-course of prostacyclin (PGI_2)-induced contraction in guinea-pig terminal ileum. W = wash.

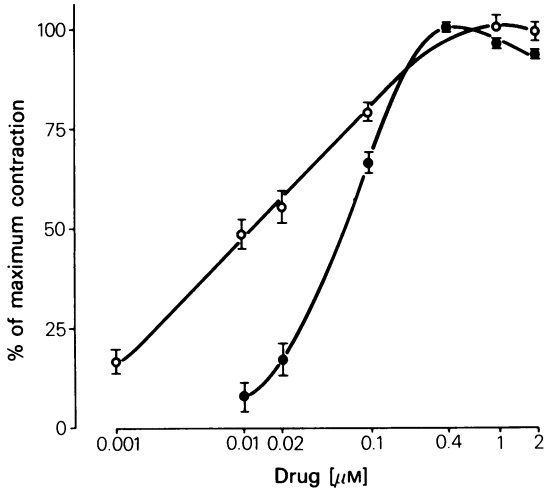

Figure 2 Non-cumulative concentration-response curves to prostacyclin (PGI_2, ○) and acetylcholine (ACh, ●) in guinea-pig terminal ileum. The response is a percentage of the maximum contraction elicited by ACh (0.4 μM). Each point is the mean of the results obtained in 8 experiments (vertical lines are s.e.).

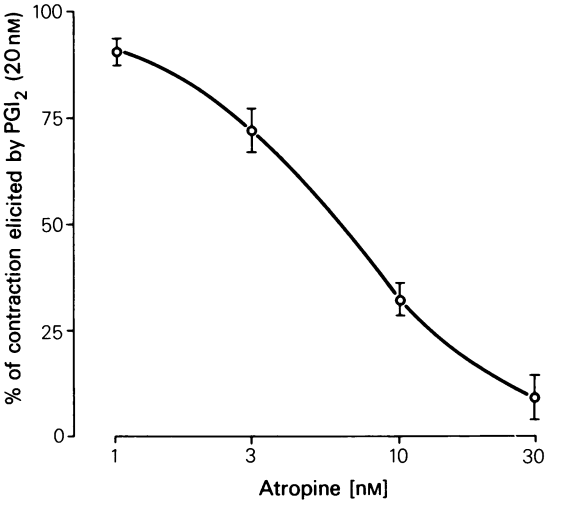

Figure 3 The concentration-dependent effect of atropine on the contraction elicited by prostacyclin (PGI_2) in guinea-pig terminal ileum. The preparations were treated with 20 nM PGI_2 in the absence and presence of different concentrations of atropine. The response is a percentage of the contraction elicited by PGI_2 in the absence of atropine. Each point is the mean of the results obtained in 5 experiments (vertical bars are s.e.).

many. Prostacyclin sodium salt was a generous gift from Carlo Erba, Italy.

Results

Effect of prostacyclin

PGI_2 elicited a contractile effect on guinea-pig ileum preparations. As shown in Figure 1, this effect was very rapid, and the tension slowly returned to normal within 10–15 min. In Figure 2 ACh and PGI_2 concentration-response curves are compared. While PGI_2 was effective at concentrations as low as 1 nM, the curve for ACh was steeper as the minimum effective concentration was 10 nM and the maximum was 0.4 μM. In the different preparations, the EC_{50} for PGI_2 ranged from 10 to 20 nM. PGI_2 saline (0.9% NaCl) solutions maintained at room temperature overnight had no effect on the ileum (not shown).

Influence of atropine on the effect of prostacyclin

The effect of PGI_2 (20 nM) was inhibited by atropine (1–30 nM) in a concentration-dependent manner (Figure 3); 30 nM atropine virtually abolished PGI_2-induced contractions (90.8% inhibition).

Table 1 The effect of physostigmine on prostacyclin (PGI_2)-induced contraction in guinea-pig terminal ileum

Pretreatment	*Treatment*	*Response* (% of maximum contraction elicited by ACh)	n	P
—	PGI_2 (20 nM)	53.85 ± 4.77	7	
Physostigmine (50 nM)	PGI_2 (20 nM)	79.18 ± 5.02	7	<0.005

The preparations were challenged with 20 nM PGI_2 in the absence and presence of physostigmine (50 nM), which was added to the bathing fluid 10 min before PGI_2. The responses were measured and calculated as a percentage of the maximum contraction elicited by ACh (0.4 μM). The indicated values are mean ± s.e.; n = number of experiments; P = significance of the difference from control calculated by Student's t test.

Influence of physostigmine on the effect of prostacyclin

Table 1 summarizes the results obtained by testing a submaximal concentration of PGI_2 (20 nM) in the absence and presence of physostigmine, an inhibitor of cholinesterase. Pretreatment of the preparations with physostigmine (50 nM) significantly potentiated the response of the ileum to PGI_2.

Influence of hemicholinium-3 on the effect of prostacyclin

Pretreatment of the preparations with hemicholinium-3 (20 μM) caused a marked decrease of the contractile response to PGI_2 (Figure 4). At all the concentrations of PGI_2 tested (1, 20 and 100 nM) the residual response in the presence of hemicholinium-3 was one third of the control contraction measured in the absence of the inhibitory drug. In the same conditions, i.e. after 30 min treatment with 20 μM hemicholinium-3, the submaximal

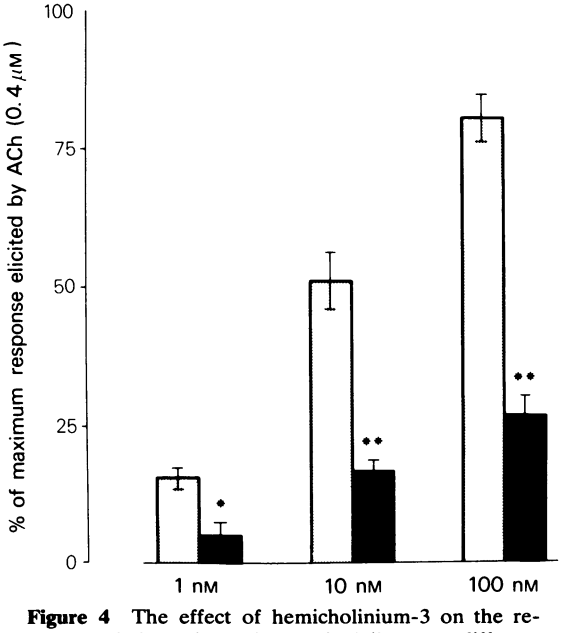

Figure 4 The effect of hemicholinium-3 on the response of the guinea-pig terminal ileum to different concentrations of prostacyclin (PGI_2). The preparations were challenged with 1, 10 or 100 nM PGI_2 in the absence (stippled columns) and presence (solid columns) of 20 μM hemicholinium-3. The results are expressed as a percentage of the maximum contraction elicited by ACh (0.4 μM). Each value is the mean of the results obtained in 4 experiments (vertical lines are s.e.). The statistical significance of the differences between hemicholinium-3-treated and non-treated preparations was calculated by Student's t test: $^*P<0.02$; $^{**}P<0.001$.

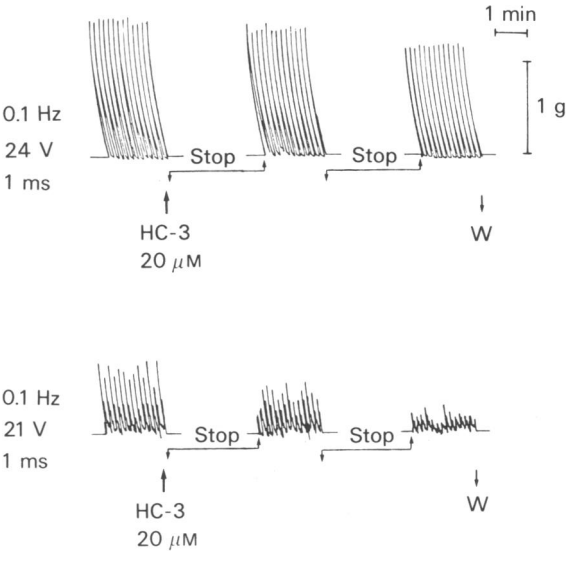

Figure 5 The effect of hemicholinium-3 (HC-3) on the response of the guinea-pig terminal ileum to maximal and submaximal electrical stimulation. Stop = stop chart and electrical stimulation for 14 min.

twitch response of the ileum to electrical stimulation was markedly inhibited, while the response to maximal stimulation was only marginally affected (Figure 5).

Influence of hexamethonium on the effect of prostacyclin

In preparations incubated in the presence of hexamethonium (100 μM) the response to submaximal PGI_2 concentrations (1 and 20 nM) was significantly lower than in control preparations (Figure 6). At maximal PGI_2 concentrations (1 μM) the effect of hexamethonium was not statistically significant (Figure 6).

Influence of β-bungarotoxin, tetrodotoxin and low temperature on the effect of prostacyclin

The muscle contraction elicited by 20 nM PGI_2 was significantly reduced when ileum preparations were exposed to β-bungarotoxin (5 μg ml^{-1}) (Figure 7). At the same concentration of PGI_2 the response was completely abolished by tetrodotoxin (10 nM) as well as by previous cooling of the ileum at 20°C for 60 min (Figure 7). These treatments did not affect the response to ACh (0.4 μM) added to the incubation medium (not shown).

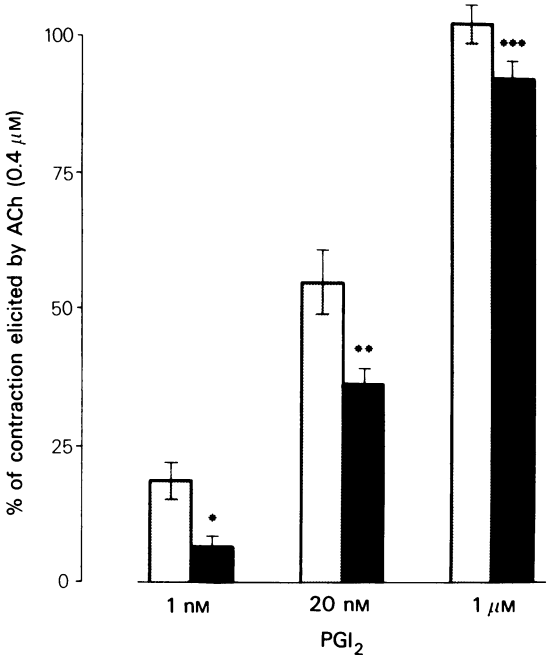

Figure 6 The effect of hexamethonium on the response of the guinea-pig terminal ileum to different concentrations of prostacyclin (PGI_2). The experiments were performed on adjacent segments of the same ileum, one of which was bathed with normal Tyrode solution (stippled columns), the other with Tyrode solution containing 100 μM hexamethonium (solid columns). The preparations were challenged with different concentrations of PGI_2 (1 nM, 20 nM, 1 μM) and the response expressed as a percentage of the maximum contraction elicited by acetylcholine (ACh, 0.4 μM). No change was observed in the response of the same preparation to ACh before and after treatment with hexamethonium for 60 min. Each value represents the mean of the results obtained in 6 experiments (vertical bars indicate s.e.). The statistical significance of the differences was calculated by Student's *t* test: $*P<0.02$; $**P<0.005$

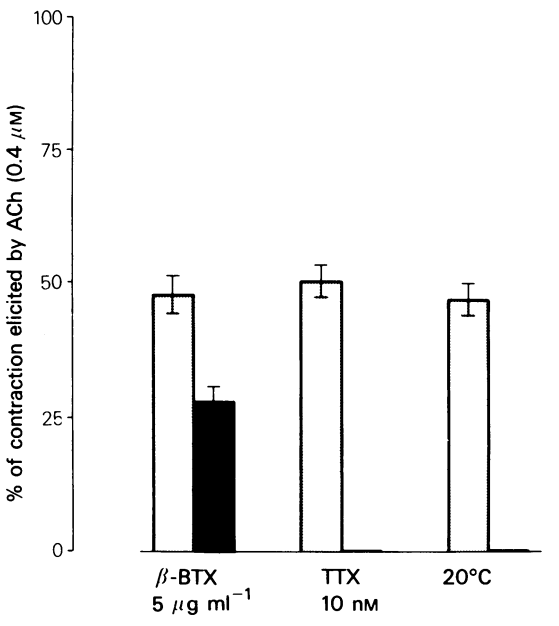

Figure 7 The influence of β-bungarotoxin (β-BTX), tetrodotoxin (TTX) and low temperature (20°C) on the contractions of the guinea-pig terminal ileum elicited by a fixed concentration (20 nM) of prostacyclin (PGI_2). The response is a percentage of the maximum contraction elicited by acetylcholine (ACh, 0.4 μM). Each value is the mean of the results obtained in 6 experiments (vertical bars indicate s.e.). In the three sets of experiments (β-BTX, TTX, 20°C) the difference between the mean values of paired determinations was highly significant ($P<0.001$) as calculated by Student's *t* test.

Discussion

The ability of PGI_2 to contract guinea-pig ileum has been shown before (Moncada *et al.*, 1976; Sirois *et al.*, 1981). According to our results the contraction

Effect of prostacyclin on the contraction of the ileum induced by electrical stimulation

As shown in Figure 8, PGI_2 potentiated the contractions induced by electrical stimulation. At concentrations of PGI_2 higher than 10 nM, a transient increase of muscle tone was also observed.

Interaction between prostacyclin and acetylcholine in tetrodotoxin-treated ileum

In preparations treated with tetrodotoxin (10 nM) the contraction elicited by a submaximal concentration of ACh (40 nM) was not significantly altered by 10 nM PGI_2 (Table 2).

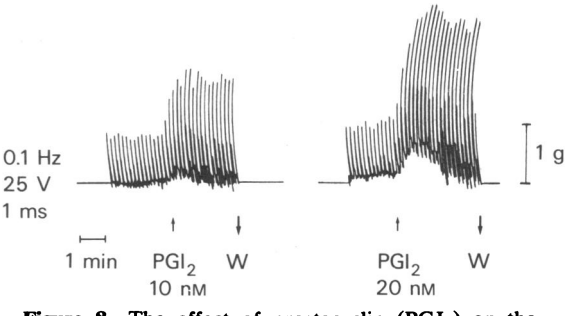

Figure 8 The effect of prostacyclin (PGI_2) on the electrically stimulated guinea-pig ileum. PGI_2 (10 and 20 nM) potentiates the twitch responses to submaximal stimulation. PGI_2 20 nM also causes a transient increase in muscle tone.

Table 2 Interaction between prostacyclin (PGI_2) and acetylcholine (ACh) in tetrodotoxin-treated preparations

Pretreatment	*Treatment*	*Response* (% of maximum contraction elicited by ACh)	n
Tetrodotoxin (10 nM)	ACh (40 nM)	61.77 ± 3.95	8
Tetrodotoxin (10 nM)	ACh + PGI_2 (40 nM) (10 nM)	56.51 ± 6.18	8

After 3 min pretreatment with tetrodotoxin, the preparations were challenged with 40 nM ACh or 40 nM ACh plus 10 nM PGI_2 (added simultaneously). The responses were measured and calculated as a percentage of maximum contraction elicited by ACh (0.4 μM). The indicated values are mean ± s.e.; n = number of experiments. The difference between the two means is not statistically significant.

elicited by PGI_2 was very rapid in onset. The progressive decrease of tension after the peak was reached may reflect the inactivation of this prostaglandin, as PGI_2 is unstable in aqueous solution and its antiaggregating activity disappears within 10 min at 37°C (Gryglewski, Bunting, Moncada, Flower & Vane, 1976). This is in agreement with our observation that PGI_2 dissolved in saline and maintained overnight at room temperature was not effective on the ileum.

The concentration-dependent inhibition of the effect of PGI_2 induced by atropine indicates that the contraction elicited by this prostaglandin is mediated by a stimulation of muscarinic receptors, since atropine is a specific antagonist of these receptors.

In our experiments physostigmine, which prevents hydrolysis of ACh by inhibiting cholinesterase activity, potentiated the effect of PGI_2, suggesting that this effect is due to the release of ACh from nerve terminals. Physostigmine, by increasing the amount of active neurotransmitter present in the synaptic cleft, would amplify the response to PGI_2.

The hypothesis that the action of PGI_2 is mediated by a presynaptic release of ACh is also supported by the results obtained in the presence of hemicholinium-3, β-bungarotoxin and tetrodotoxin.

Hemicholinium-3 is known to inhibit choline uptake into nerve endings in a competitive manner, thus hindering the presynaptic synthesis and release of ACh (Bhatnagnar, Lam & McColl, 1964). Therefore the response to drugs which act by stimulating the presynaptic release of ACh will be inhibited by hemicholinium-3. This was also the case for contractions induced by PGI_2 as well as by submaximal electrical stimulation in the ileum. On the other hand, the maximum twitch response of the preparations to electrical stimulation was much more resistant to the inhibitory effect of hemicholinium-3, as previously observed by Down & Szerb (1980). This seems to indicate that the effect of PGI_2 would be mimicked better by the application of a submaximal, rather than a maximal, current voltage.

Tetrodotoxin, by acting on sodium channels, blocks the conduction of action potentials in nerve cells, without affecting the electrical activity of smooth muscle cells (Gershon, 1967). These properties make tetrodotoxin a good tool for identifying the nerve-mediated effects of drugs. Pretreatment of the preparations with tetrodotoxin abolished the effect of PGI_2 on the ileum, further indicating that this effect is not due to a direct action on smooth muscle, but is mediated by nerve stimulation. It is also known that tetrodotoxin does not prevent ACh release induced by direct depolarization of nerve terminals (Paton, Vizi & Zar, 1971) and it is thus likely that PGI_2 acts on cholinergic cell bodies of the myenteric plexus rather than on presynaptic nerve endings.

It has been clearly demonstrated that β-bungarotoxin abolishes ACh release not only in somatic motor nerves, but also in parasympathetic fibres (Miura, Muramatsu, Fujiwara, Hayashi & Lee, 1981). There are, however, species and regional differences in the parasympathetic blocking action of this toxin, guinea-pig ileum being only partially sensitive to it (Miura *et al.*, 1981). This would agree with the fact that, in our experiments, β-bungarotoxin reduced, but did not abolish the contractions elicited by PGI_2.

The ganglionic blocker hexamethonium has been shown to act as a competititive antagonist on nicotinic receptors in guinea-pig ileum, without affecting transmitter release (Hayashi, Yamada & Mori, 1977).

Hexamethonium partially depressed the response of the ileum to submaximal, but not maximal PGI_2 concentrations. This indicates that a portion of PGI_2-induced contraction is secondary to activation of ganglionic nicotinic receptors, possibly due to stimulation of cholinergic intrinsic interneurones, as the number of extrinsic fibres is negligible as compared to the enteric ones (Ambache, 1955; Kosterlitz & Lees, 1964). Similarly, Yagasaki *et al.* (1981) found that in guinea-pig myenteric plexus-longitudinal

muscle preparations the release of ACh evoked by PGE_1 was abolished by tetrodotoxin and only reduced by hexamethonium.

Further evidence for the involvement of endogenous ACh in PGI_2-induced contractions is given by the results obtained in ileum preparations maintained at 20°C. Lowering the temperature of the bath fluid affects all nervous structures of the ileum, thus hindering the release of ACh from presynaptic nerve terminals and, even more markedly, at the ganglionic level (Innes, Kosterlitz & Robinson, 1957; Kosterlitz & Lees, 1964). This may explain the lack of effect of PGI_2 in preparations incubated at 20°C.

Finally the ability of PGI_2 to potentiate the contractions elicited by electrical stimulation with external surface electrodes further agrees with the nerve-mediated effect of this prostaglandin. In fact this potentiation cannot be ascribed to a direct action of PGI_2 on smooth muscle, as PGI_2 did not alter the response to exogenous ACh in tetrodotoxin-treated preparations.

The mechanism of action of PGI_2 would thus be similar to that of prostaglandins of the E series, namely PGE_1 and PGE_2, which have been shown to facilitate ACh outflow in guinea-pig ileum by acting on intramural nerves (Kadlec *et al.*, 1978; Yagasaki *et al.*, 1980).

There is, however, an apparent discrepancy between this interpretation and the early study of Moncada *et al.* (1976) showing the contractile effect of PGI_2 on the ileum, as in that study the cascade superfusion technique (Vane, 1964) was used to test the activity of this new prostaglandin and a mixture of antagonists (Gilmore, Vane & Wyllie, 1968), including a muscarine receptor antagonist, was added to the Krebs solution superfusing the preparations. Very recently prostanoid receptors in a number of smooth muscle preparations have been classified on the basis of the rank order of agonist potency of naturally occurring prostanoids (Kennedy, Coleman, Humphrey, Levy & Lumley, 1982). Despite the presence of atropine in the solution bathing the preparations, PGE_1, PGE_2, PGI_2 as well as other prostaglandins contracted the guinea-pig ileum. However, the reported ED_{50} of PGI_2 was 2.7 μM, which is more than 100 times higher than the one estimated from our experiments. One possible explanation for these discrepancies is that two different types of prostanoid receptors are present in the guinea-pig ileum, one of which is located on smooth muscle cells, the other on the neurones of the myenteric plexus. The latter type would be sensitive to very low, physiological concentrations of PGI_2, which elicit indirect effects on the ileum, while higher concentrations would be required to stimulate postsynaptic receptors, with a consequent atropine-resistant contraction of the ileum.

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