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THE EFFECT OF INDOMETHACIN ON THE RELEASE OF PROSTAGLANDIN E_2 AND ACETYLCHOLINE FROM GUINEA-PIG ISOLATED ILEUM AT REST AND DURING FIELD STIMULATION

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1 Guinea-pig ileum suspended in Krebs solution showed a continuous increase of tone which was lost by changing the bath fluid. Prostaglandin E_2 was released from the ileum during incubation, and its concentration in the bath fluid appeared to correlate with the increase in tone.

2 Supramaximal field stimulation (10 Hz) resulted in increased release of prostaglandin E_2 from the ileum. At lower rates of stimulation, the increase in the release of E_2 compared with the resting output was not significant.

3 Indomethacin (1 and 10 μg/ml) produced a significant, dose-related reduction of the amount of prostaglandin E_2 measured in the bath fluid at rest and with field stimulation. Indomethacin inhibited the contraction of the ileum during incubation in Krebs solution.

4 Indomethacin (10 and 20 μg/ml) had no significant effect on the release of acetylcholine during field stimulation, but reduced the resting output of acetylcholine from guinea-pig ileum in some experiments.

5 The results are discussed in the context of the role ascribed to prostaglandins as physiological modulators in transmitter release. No evidence for a prostaglandin-mediated negative feedback mechanism on acetylcholine release was obtained.

Introduction

Of the many effects of the prostaglandins (Bergström, Carlson & Weeks, 1968; Ramwell & Shaw, 1970; Horton, 1972), interest has recently been focused on the role of this class of compounds in the autonomic nervous system. Thus, according to Davies, Horton & Withrington (1968) and Gilmore, Vane and Wyllie (1968), stimulation of the splenic sympathetic nerves in the dog and in the cat causes a release of prostaglandins E_2 and $F_{2\alpha}$ from the spleen. Administration of prostaglandins E_1 and E_2 inhibits the release of noradrenaline induced by sympathetic nerve stimulation in isolated cat spleen (Hedqvist & Brundin, 1969; Hedqvist, 1970), inhibits the effects of sympathetic stimulation in rabbit heart by reducing the release of the transmitter noradrenaline from the adrenergic nerve endings (Hedqvist, Stjärne & Wennmalm, 1970; Wennmalm & Hedqvist, 1970; Hedqvist & Wennmalm, 1971; Wennmalm, 1971; Wennmalm and Stjärne, 1971), and interferes with effector response to sympathetic nerve stimulation in the vas deferens of guinea-pigs (Ambache & Zar, 1970; von Euler & Hedqvist, 1969). This type of effect has not been found with prostaglandin $F_{2\alpha}$ on dog spleen (Davies & Withrington, 1971) and rabbit heart (Hedqvist & Wennmalm, 1971). The finding that prostaglandins of the E series inhibit the release of noradrenaline led to the hypothesis that prostaglandins act as inhibitors (physiological modulators) in a feed-back system which regulates the release of noradrenaline caused by nerve stimulation. The observation that the inhibition of prostaglandin synthesis results in an increased release of noradrenaline is further evidence for the participation of prostaglandins in an endogenous control mechanism (Samuelsson & Wennmalm, 1971; Wennmalm, 1971).

The results of Wennmalm & Hedqvist (1971) indicate that prostaglandin E_1 inhibits the chronotropic response to stimulation of the vagal cardiac nerves in rabbits. As a result of these findings it is postulated that exogenous prostaglandin E_1 has an inhibitory effect on the release

of cholinergic transmitter in the same way as it does in the sympathetic nervous system, although there is no direct evidence for this at present. We therefore felt it would be interesting to examine the effects of an inhibitor of prostaglandin synthesis on release of acetylcholine (ACh) from parasympathetic nerves, in the field stimulated guinea-pig isolated ileum preparation.

Methods

Guinea-pig isolated ileum

Male guinea-pigs weighing from 370 to 850 g (mean weight 570 g) were killed by a blow on the neck. The ileum pieces were placed in 5 and 8 ml organ baths containing Krebs solution gassed with 95% O_2, 5% CO_2 mixture. The composition of the Krebs solution was as follows (g/litre distilled water): NaCl, 7.0; $NaHCO_3$, 2.1; glucose, 2.0; KCl, 0.35; $MgSO_4 \cdot 7H_2O$, 0.14; KH_2PO_4, 0.16; $CaCl_2 \cdot 2H_2O$, 0.289. For the tests to determine ACh release, the Krebs solution contained physostigmine sulphate 2 μg/ml (Paton & Zar, 1968; Paton & Vizi, 1969). In some experiments, the Krebs solution contained indomethacin (1-20 μg/ml). The bath temperature was 37° C. Movements of the ileum were recorded with a frontal recorder on a smoked kymograph (magnification 1 : 7, load 1.5 g). Before beginning the test, the ileum was left in the bath for 1 to 1.5 h to reach equilibrium, during which time the bath fluid was repeatedly changed. The period allowed for accumulation of prostaglandin or ACh was usually 15 minutes. In some tests, the rest period was shortened to 10 min or increased to a maximum of 120 min, and the duration of the stimulation period was shortened to 10 min or prolonged to 20 minutes. After the appropriate times, the bath contents were withdrawn with a syringe and tested for prostaglandin-like activity and ACh. Field stimulation was applied with rectangular impulses of 0.7 ms duration, frequencies from 0.2 to 10 Hz at supramaximal voltage.

Prostaglandin determination

The bath samples from guinea-pig isolated ileum were tested for prostaglandin-like activity by the method described by Gilmore *et al.* (1968) and Vane (1971, 1972) in a superfusion test on rat stomach strip and rat colon. To increase the specificity of the method, as recommended by Gilmore *et al.* (1968), the Krebs solution contained hyoscine, mepyramine and methysergide (0.1 μg/ml in each case). The contractions of the rat stomach strip and rat colon were recorded with frontal recorders on a smoked kymograph (magnification 1 : 16 and load 4 g for rat stomach strip; magnification 1 : 18 and load 2 g for rat colon) or recorded on a Devices pen-recorder.

Prostaglandin activity was assayed by comparison of its effects with those of known standard doses of prostaglandin E_2. Where no prostaglandin activity could be detected (as sometimes occurred in the presence of indomethacin), the content was recorded as equivalent to the smallest amount of prostaglandin E_2 that could be detected by the assay.

In some experiments, the bath fluid surrounding the guinea-pig small intestine was collected at 15 min intervals and pooled (total volume up to 100 ml). This was adjusted to pH 3.5 with 1 M hydrochloric acid and extracted twice with an equal volume of ethyl acetate (Gilmore *et al.*, 1968). The ethyl acetate phase was evaporated to dryness under reduced pressure at 50° C and the residue taken up in 1 ml Krebs solution. Thin-layer chromatography was carried out by the method described by Willis (1970). The extracted test solution and the prostaglandin $F_{2\alpha}$, E_1 and E_2 reference solutions were applied to the left and right halves of the plates, in each case with the same arrangement. Only the spots on one half of the plate were visualized. The layer of silica gel on the other half of the plate was divided up into various zones, from which the silica gel layers were carefully removed, shaken in 1 ml Krebs solution and centrifuged for 5 minutes. The supernatant solutions were then tested for prostaglandin-like activity on the rat stomach strip and rat colon.

Acetylcholine determination

ACh was determined by the method described by Paton & Zar (1968) and Paton & Vizi (1969) on guinea-pig isolated ileum. The organ bath contained Krebs solution with morphine sulphate (10 μg/ml) (Paton, 1957) and physostigmine sulphate (5 ng/ml) and was aerated with 5% CO_2 and 95% O_2. To increase the specificity, methysergide (0.1 μg/ml) was added to the fluid. The contractions were recorded on the smoked kymograph with a frontal recorder (magnification 1 : 7 to 1 : 14, load 1.5 g). A small volume (0.1 to 0.4 ml) of the bath fluid was added to the organ bath for determination of ACh and the contractions were compared with those produced by known doses of ACh.

Results

After only a few minutes incubation in Krebs solution, the guinea-pig ileum showed a definite

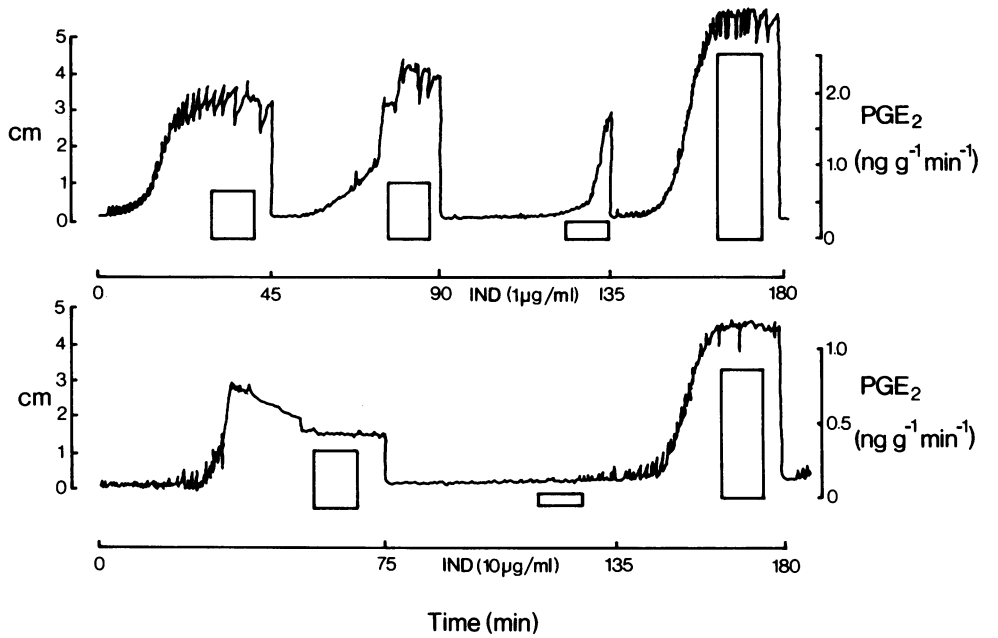

Fig. 1 The correlation between increase of tone in guinea-pig ileum and the release of prostaglandin E_2 (PGE_2), the effect of indomethacin, 1 and 10 μg/ml is also shown. The ileum was allowed to rest for periods of 45 to 75 min. Columns represent amount of prostaglandin E_2. IND = indomethacin.

and continuous increase in tone. On changing the bath fluid, the tone of the ileum returned to that recorded at the beginning of the rest period (Figure 1).

Bath fluid, sampled after each rest period, exhibited prostaglandin-like activity on the rat stomach strip. It had no effect on the rat colon. The properties of the substance released coincided with those of prostaglandin E_2 on thin-layer chromatography and with those of standard prostaglandin E_2 on rat stomach strip and rat colon.

The average rate of prostaglandin E_2 release was 0.6 ± 0.1 ng g^{-1} min^{-1} (27 samples from 12 experiments). Release of prostaglandin E_2 paralleled the contraction of the guinea-pig ileum during rest periods (Figure 1).

The effects of 1 and 10 μg/ml indomethacin on tone and prostaglandin E_2 release in guinea-pig ileum was studied in six experiments. Figure 1 shows two representative recordings from guinea-pig small intestine which had been treated with 1 and 10 μg/ml indomethacin. Indomethacin 1 μg/ml abolished almost completely the increase of tone in the tissue; the ileum contracted just before the end of the period of contact with indomethacin. Indomethacin 10 μg/ml completely abolished the increase of tone in the tissue, and spontaneous activity of the ileum, which is sometimes observed in the resting period, was reduced or completely suppressed. Whilst the tissue was in contact with indomethacin, there was a reduction in the amount of prostaglandin E_2 in the bath fluid and, in a number of tests, the reduction was to levels below the measurable limit. After washing out the indomethacin, the contraction of the ileum was invariably greater and even more prostaglandin E_2 was released than in the pre-indomethacin resting phase (Figure 1). The results of all the experiments are summarized in Figure 2.

Field stimulation

Supramaximal field stimulation increased release of prostaglandin E_2 from guinea-pig ileum, the amount released depending on the stimulus frequency (Figure 2). The amount of prostaglandin found after stimulation at 0.2 Hz was no greater than with unstimulated tissue; with 1 Hz, the small increase in prostaglandin concentration was not statistically significant. A stimulus frequency of 10 Hz was required to produce a significant ($P = 0.025$) increase of prostaglandin release compared with the resting output.

There was a difference between contractions produced at different stimulus frequencies. With frequencies below 1 Hz, 'spike' contractions were

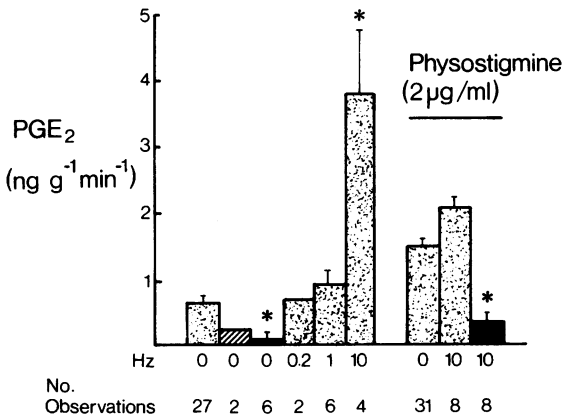

Fig. 2 The action of indomethacin on output of prostaglandin E_2 (PGE_2) from normal and physostigmine-treated guinea-pig isolated ileum at rest and during field stimulation. Hz – stimulus frequency; diagonally hatched bars – indomethacin 1 μg/ml; solid bars – indomethacin 10 μg/ml. Standard error indicated by the vertical line. * Significant at $P = 0.05$ or less.

produced, whereas frequencies of 1 and 10 Hz produced a rapid contraction which then fell to about 50% of the initial height and the muscle remained at that degree of shortening throughout the stimulation period. The size and pattern of the contractions were the same at 1 and 10 Hz, indicating that maximal responses were produced with the lower frequency. Even so we were able to demonstrate a definite increase of prostaglandin release only at 10 Hz.

The release of prostaglandin E_2 elicited by field stimulation with 10 Hz was reduced to below measureable limits by indomethacin (10 μg/ml) (Figure 2).

In two experiments, the effect of indomethacin (10 μg/ml) on the contractions produced by 0.2 Hz field stimulation, and on the contraction to exogenous ACh, was examined. Apart from causing a small, transient reduction of the effect of field stimulation and of the action of ACh, indomethacin did not cause any change in the two responses.

Preparations containing physostigmine

The physostigmine-treated guinea-pig ileum is in a state of permanent contraction. It is therefore impossible to make the comparison between tone and prostaglandin release which was described in the absence of physostigmine. In these experiments, there was a greater release of prostaglandin E_2 than in the absence of physostigmine (1.54 ± 0.2 compared with 0.6 ± 0.1 ng g^{-1} min^{-1}, see Figure 2). Unlike the situation in the absence of physostigmine, the release of prostaglandin E_2 with field stimulation (10 Hz) was not significantly higher than the resting output (see Figure 2). Also, in contrast to the response of the ileum which was not treated with physostigmine, addition of indomethacin (1, 5 and 10 μg/ml) produced only partial, or no relaxation of the organ although the amount of prostaglandin E_2 measured in the bath fluid was significantly reduced by 10 μg/ml indomethacin at rest and stimulation (Figure 3).

The effect of indomethacin on the release of acetylcholine at rest and during field stimulation

The output of ACh from guinea-pig ileum at rest varied in different experiments from 18 to 72 ng g^{-1} min^{-1}. Indomethacin (10 or 20 μg/ml) always caused a slight reduction in this output, although in only two experiments was this significant (Figure 3). Field stimulation increased the output of ACh, the greatest output (per pulse) being observed at low frequencies of stimulation. Indomethacin (10 or 20 μg/ml) was without significant effect on release of ACh in response to field stimulation although in all but one case a small reduction was observed (Figure 3). The diluent used for the indomethacin also had no influence on the release of ACh during nerve stimulation (Figure 3).

The spasmogenic activity in the fluid of the organ bath was detected and assayed on the guinea-pig isolated ileum treated with physostigmine (in the presence of morphine, 10 μg/ml) and was blocked by hyoscine (1 ng/ml). The aqueous residues remaining after acidification and extraction with ethyl-acetate appeared to contain concentrations of ACh equal to non-extracted samples, indicating that the prostaglandin content did not interfere with the assay of ACh.

Discussion

Our experiments have shown that, when incubated in Krebs solution, the guinea-pig ileum develops tone and that prostaglandin E_2 is released. It is probable that the release of prostaglandin is directly responsible for the increase of tone in the tissue, since this was prevented by inhibition of prostaglandin synthesis induced by indomethacin (Vane, 1971, 1972). Thus, our results obtained with guinea-pig ileum agree with those of Ferreira, Herman & Vane (1972), and Davison, Ramwell & Willis (1972) who found that prostaglandins maintain the muscular tone in rabbit jejunum and guinea-pig ileum respectively. After the inhibitory

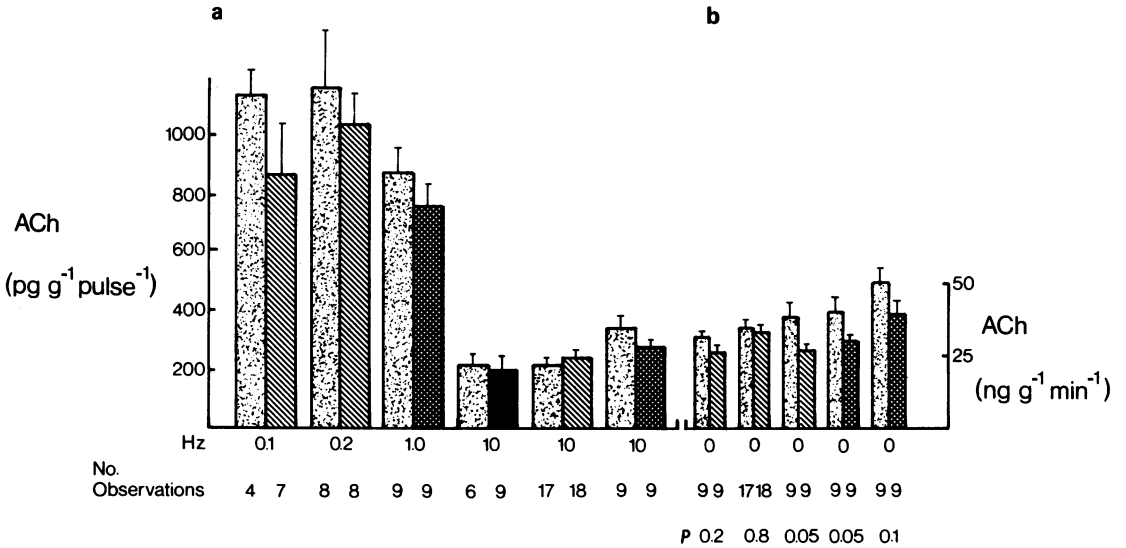

Fig. 3 The effect of indomethacin on output of acetylcholine (ACh) from guinea-pig isolated ileum (a) during field stimulation and (b) at rest. (Diagonal hatching — indomethacin 10 μg/ml; crossed diagonal hatching — indomethacin 20 μg/ml; solid column — in presence of diluent for indomethacin). *P* = probability level (*t*-test).

action of indomethacin had declined, we found that the ileum had a higher tone than before indomethacin administration, and this was possibly related to the observed increase in release of prostaglandin E_2.

Field stimulation with high frequencies produced a measurable increase in release of prostaglandin E_2 in the ileum which was not treated with physostigmine. The fact that frequencies of 1 and 10 Hz elicit practically the same level of contraction of the effector organ, but that larger quantities of prostaglandin E_2 are found only with a stimulus frequency of 10 Hz, suggests that prostaglandin E_2 is released primarily as a result of nerve stimulation at that frequency and is not extruded from the intestine by the mechanical effect of the contraction of smooth muscle. It is at a field stimulation frequency of 10 Hz that noradrenaline is released from guinea-pig isolated ileum (Hughes, personal communication). Thus, it is possible that it is catecholamines which are primarily responsible for release of prostaglandin in this preparation. On the other hand, the elevated resting output of prostaglandin E_2 in the organ treated with physostigmine compared with the resting output of untreated ileum may be due to the extremely powerful physostigmine-induced contraction of the ileum. In these preparations, the stimulus frequency of 10 Hz did not induce a significant increase of prostaglandin E_2, suggesting that, in the presence of physostigmine, synthesis was occurring at a rate too fast to be further increased by stimulation.

The experiments were not designed to establish the source of the prostaglandin E_2. Further experiments are planned to elucidate this using the longitudinal muscle strip preparation described by Paton & Vizi (1969).

Prostaglandins are ascribed the role of modulators in the release of transmitter in the adrenergic nervous system (Samuelsson & Wennmalm, 1971; Wennmalm, 1971). It has been suggested that there may be a similar feed-back mechanism for the parasympathetic nervous system (Wennmalm & Hedqvist, 1971). If this type of feed-back mechanism actually existed in the parasympathetic nervous system, we might expect to find an increase in ACh release in our guinea-pig isolated ileum preparation when it is treated with indomethacin, because this would abolish any inhibitory action of prostaglandin. Indomethacin indeed caused a marked reduction of prostaglandin E_2 release but, in spite of this pronounced inhibition of synthesis, the ACh content was unchanged, or slightly reduced at rest and during field stimulation at various frequencies. An alternative, to the use of indomethacin to inhibit the synthesis of prostaglandins in this investigation, would have been to have measured ACh release in the presence of high concentrations of

exogenous prostaglandin E_2. However, this would carry the assumption that it is prostaglandin E_2 that is solely involved in any feedback mechanism, whereas it may be another prostaglandin not detected by the standard bioassay methods, or even two or more prostaglandins acting in combination. For these reasons inhibition of synthesis of endogenous prostaglandin was preferred.

Thus, we did not find any evidence of a prostaglandin-mediated negative feedback mechanism affecting ACh release with the test preparation and method we used. However, it should be pointed out that, even with the high dose of 20 μg/ml indomethacin, the synthesis of prostaglandin E_2 might not have been completely abolished, the measurements being circumscribed by the limits of sensitivity of the methods used. It is possible therefore that prostaglandin synthesis was maintained at a certain minimal level and it may be that only a very small quantity of endogenous prostaglandin would be necessary to inhibit maximally the release of ACh at rest and with field stimulation.

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