The differential contribution of endogenous prostaglandins to the release of acetylcholine from the myenteric plexus of the guinea-pig ileum

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1 Prostaglandin E (PGE) may be essential for maintaining the sensitivity of the myenteric plexus of guinea-pig ileum to nicotine. The contributions of prostaglandins to nervous activity evoked by different stimuli have now been investigated by measuring the amount of acetylcholine (ACh) released from the myenteric plexus of the guinea-pig ileum.

2 The amount of ACh released in response to dimethylphenylpiperazinium (DMPP) or substance P was depressed to about 40% of control by 2.8 μ M indomethacin (Ind), whereas the release of ACh induced by 5-hydroxytryptamine (5-HT) was not affected. The inhibitory effects of Ind were overcome by 14.3 nM PGE₂.

3 Mepacrine 5 μ M, an inhibitor of phospholipase A₂, depressed the release of ACh in response to DMPP and substance P to the same extent as Ind. These inhibitory effects of mepacrine were overcome by arachidonic acid (10 μ M), but not by arachidonic acid plus Ind. The release of ACh evoked by 5-HT or electrical field stimulation (EFS) was also inhibited to about 60% of control by mepacrine but these inhibitions were overcome by arachidonic acid (10 μ M) either in the absence or the presence of Ind.

4 The results suggest that endogenous prostaglandins and arachidonic acid contribute to the maintenance of the excitability of the myenteric plexus by DMPP and substance P. By contrast, the release of ACh induced by 5-HT and EFS may be regulated by arachidonic acid and not by prostaglandins.

Introduction

In the myenteric plexus of the guinea-pig ileum, the release of acetylcholine (ACh) evoked by electrical stimulation or a high concentration of K⁺, which depolarize the axons or terminals of neurones, respectively, is not affected by 2.8 μ M indomethacin (Ind), an inhibitor of cyclo-oxygenase (Yagasaki *et al.*, 1984). Contradictory results have been obtained in studies with nicotine, which has been shown to activate the soma of myenteric neurones (Yagasaki *et al.*, 1984; Wood, 1987). Ind inhibited both the release of ACh from the myenteric plexus and the response of the enteric inhibitory nerves in the taenia coli to nicotine (Okpako & Taiwo, 1984; Yagasaki *et al.*, 1984; Iselin *et al.*, 1988). Such inhibition was overcome by prostaglandin E₂ (PGE₂). In addition, the inhibition of phospholipase A₂ by mepacrine, which decreases the liberation of arachidonic acid, depressed the excitability of cholinergic neurones by nicotine in the same manner as did Ind, and this inhibition was overcome by arachidonic acid (Takeuchi & Yagasaki, 1987). Since the restorative effect of arachidonic acid on the inhibition by mepacrine was partly overcome by Ind, the effect of arachidonic acid was assumed to be due to both arachidonic acid itself and to prostaglandins synthesized from exogenously added arachidonic acid (Takeuchi & Yagasaki, 1987). These pieces of evidence suggest that the products of the arachidonic acid cascade, in particular arachidonic acid and PGE₂, may contribute to the modulation of the excitability of the myenteric neurones by nicotine.

Substance P and 5-hydroxytryptamine (5-HT) may be present in the myenteric plexus (Llewellyn-Smith *et al.*, 1989; Costa *et al.*, 1982), to excite the somal membranes of enteric neurones (Wood, 1987). The application of these agents to guinea-pig ileum induces the release of ACh via direct stimulation of the cholinergic neurones (Holzer & Lembeck, 1980; Kilbinger & Pfeuffer-Friederich, 1985). However, the contribution of prostaglandins to the release of ACh induced by substance P and 5-HT remains to be examined. In order to determine whether or not a common mechanism, sensitive to the products of the arachidonic acid cascade, is involved in the receptor-mediated release of ACh, we determined the effects of Ind and mepacrine on the release of ACh induced by

substance P and 5-HT. The results were compared with the effects of inhibitors of arachidonic acid cascade on the release of ACh induced by the nicotinic stimulant, dimethylphenylpiperazinium (DMPP) (Yokoyama *et al.*, 1986). A summary of this work was published previously (Takeuchi *et al.*, 1987).

Methods

Male guinea-pigs, weighing 300 to 700 g, were killed by a blow on the head and bled. The longitudinal muscle of the ileum with the myenteric plexus attached was prepared as previously described (Yagasaki *et al.*, 1981). The preparation was mounted in an organ bath that contained 3 ml of Tyrode solution of the following composition (mM): NaCl 136.9, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.05, NaHCO₃ 11.9, NaH₂PO₄ 0.4 and glucose 5.6. Physostigmine salicylate (5 μ M) and choline chloride (1 μ M) were added to the bathing medium, which was kept at 37°C and bubbled with 95% O₂ and 5% CO₂.

Release of acetylcholine induced by various stimuli

Before samples of bathing medium were collected for estimation of the release of ACh, the strips were equilibrated for 15 min by perfusion with Tyrode solution at a rate of 1–2 ml min⁻¹. Thereafter, the perfusion was stopped and the bathing medium was removed and replaced by 3 ml of fresh Tyrode solution at intervals of 1 min. After two consecutive samples were collected for the measurement of spontaneous release of ACh, the strips were stimulated for 1 min by addition of DMPP (10 μ M), substance P (1 μ M), 5-HT (2.6 μ M), or by electrical field stimulation (EFS). Each stimulus was applied twice with an interval of 19 min between stimuli. The first stimulation was carried out in the absence (S₁) and the second was carried out in the presence (S₂) of the drugs. For the EFS-induced release of ACh, the stimulation was performed in trains of 20 s, and bathing fluid was collected after a further 40 s resting period. EFS was carried out with a pair of platinum electrodes, one at the top and one at the bottom of the strip. The parameters for EFS were as follows: supramaximal

voltage (50 V); 0.5 ms duration; 200 pulses in all, at 10 Hz. All samples collected were kept on ice until assayed (within 3 h). At the end of the experiment, the strips were blotted and weighed to express the release as the amount of ACh per g tissue. The amount of ACh released in response to each stimulation was calculated by subtraction of the output during the immediately preceding resting period of 1 min from the total output during the period of stimulation. All results, in both the figures and in the text, are given in terms of relative amounts of ACh released. Thus, the release of ACh during S_2 is expressed as a percentage of the amount of ACh released during S_1 .

Assay of released acetylcholine

The collected samples were assayed for ACh with isolated strips of longitudinal muscle obtained from the most distal region of the guinea-pig ileum, as previously described (Takeuchi & Yagasaki, 1987). The medium in the assay bath was kept at 37°C and bubbled with 95% O_2 and 5% CO_2 . To eliminate the possibility of interference by DMPP in the collected samples, hexamethonium chloride (0.1 mM) was added to the Tyrode solution for the assays. When substance P was present in the samples, interference was prevented by treating the samples with one-tenth volume of α -chymotrypsin (50 u ml^{-1}) for 20 min at 37°C. When samples containing 5-HT were assayed, 5-HT ($2.6 \mu\text{M}$) was always present in the solution for the assays. To eliminate the possibility of interference in the assay by the other drugs present in the collected samples, the responses to the bathing fluids were compared with those to a standard solution of ACh to which the tested drugs were added, at the same final concentration.

The active substance in the collected samples was identified as ACh by demonstrating that it was destroyed by boiling in alkali and that its action was antagonized by atropine and potentiated by physostigmine.

Statistical analyses were performed by Student's *t* test.

Drugs

The following drugs were used: acetylcholine chloride (ACh), indomethacin (Ind), physostigmine salicylate, mepacrine dihydrochloride, arachidonic acid, 5-hydroxytryptamine creatinine sulphate (5-HT), α -chymotrypsin (Sigma, U.S.A.), substance P (Peptide institute, Japan), choline chloride, hexamethonium chloride dehydrate (Wako, Japan), morphine hydrochloride (Takeda Chemical Industries, Japan), tetrodotoxin (Sankyo, Japan), and dimethylphenylpiperazinium iodide (DMPP; Aldrich, U.S.A.). Prostaglandin E_2 was a gift from the Ono Pharmaceutical Company (Japan).

All other chemicals were of analytical grade. The drugs were dissolved in distilled water with the exception of indomethacin and prostaglandin E_2 which were dissolved in ethanol as stock solutions. Dilutions were made in Tyrode solution. Ethanol at the concentration used did not affect the release of ACh.

Results

In control experiments, no significant differences were observed between the first (S_1) and the second (S_2) stimulation in terms of the release of ACh induced by each stimulus used in this study. The values for the relative amounts of ACh released (S_2/S_1) were 115.6 ± 8.2 , 94.4 ± 3.5 , 110.8 ± 10.9 and $115.8 \pm 6.7\%$ for DMPP ($10 \mu\text{M}$), substance P ($1 \mu\text{M}$), 5-HT ($2.6 \mu\text{M}$) and EFS (50 V, 10 Hz and 0.5 ms for 20 s), respectively. The actual amounts of ACh released in response to various stimuli were also similar (not shown).

Release of acetylcholine induced by dimethylphenylpiperazinium

DMPP stimulates the release of ACh through the nicotinic receptors in myenteric neurones (Yokoyama *et al.*, 1986). The release of ACh evoked by DMPP ($10 \mu\text{M}$) was inhibited by Ind at $2.8 \mu\text{M}$ (to about 39% of control; Figure 1). The release was restored to 86% of control by addition of PGE_2 at 14.3 nM which itself had almost no effect on the release of ACh (Figure 1). $PGF_{2\alpha}$ 14.1 nM was ineffective (not shown). We did not use $PGF_{2\alpha}$ at concentrations higher than 14.1 nM , since we reported previously that the inhibitory effect of Ind on the nicotine-induced contraction was not affected by $PGF_{2\alpha}$ up to 141 nM (Yagasaki *et al.*, 1984). Mepacrine at $5 \mu\text{M}$ inhibited the DMPP-induced release of ACh to the same extent as Ind, and this inhibition was partially overcome by the addition of arachidonic acid at $10 \mu\text{M}$ (Figure 1). When Ind at $2.8 \mu\text{M}$ was used in addition to mepacrine, the restorative effect of arachidonic acid was eliminated (Figure 1). The release of ACh induced by DMPP was inhibited almost completely by 300 nM tetrodotoxin (TTX) (not shown).

Release of acetylcholine induced by substance P

Ind at $2.8 \mu\text{M}$ caused a significant inhibition of the release of ACh evoked by substance P ($1 \mu\text{M}$) to about 36% of control (Figure 2). This inhibitory effect of Ind was partially overcome by PGE_2 14.3 nM (Figure 2), but not by $PGF_{2\alpha}$ 14.1 nM (not shown). Mepacrine at $5 \mu\text{M}$ also depressed the release of ACh in response to substance P to about 40% of control (Figure 2). The inhibitory effect of mepacrine was partially overcome by arachidonic acid and this recovery was also eliminated by $2.8 \mu\text{M}$ Ind (Figure 2). TTX (300 nM) completely inhibited the stimulatory effect of substance P on the release of ACh (not shown).

Release of acetylcholine induced by 5-hydroxytryptamine

Ind at $2.8 \mu\text{M}$ failed to inhibit the release of ACh induced by 5-HT at $2.6 \mu\text{M}$ (Figure 3) but mepacrine at $5 \mu\text{M}$ was still effective (Figure 3). This inhibitory effect of mepacrine was overcome to a large extent by arachidonic acid at $10 \mu\text{M}$ (the release of ACh returned to about 88% of control; Figure 3). The recovery with arachidonic acid was not affected by $2.8 \mu\text{M}$ Ind. TTX (300 nM) inhibited incompletely but significantly, to $36.3 \pm 8.7\%$ of control, the release of ACh induced by 5-HT.

Figure 1 The inhibitory effects of indomethacin (Ind) and mepacrine (Mepac) on dimethylphenylpiperazinium (DMPP)-induced release of acetylcholine (ACh) from the myenteric plexus of guinea-pig ileum and the reversals of these effects by prostaglandin E_2 (PGE_2) and arachidonic acid (AA). DMPP was present at $10 \mu\text{M}$. Ind, Mepac and AA were added to the bath 17 min before S_2 and PGE_2 was 2 min before S_2 . The columns show the means with s.e. shown by vertical bars. Numbers of experiments are shown in parentheses. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with controls. ^a $P < 0.01$ as compared with Ind. ^b $P < 0.05$ as compared with Mepac and AA.

Figure 2 The inhibitory effects of indomethacin (Ind) and mepacrine (Mepac) on substance P-induced release of acetylcholine (ACh) from the myenteric plexus of guinea-pig ileum and the reversals of these effects by prostaglandin E₂ (PGE₂) and arachidonic acid (AA). Substance P was present at 1 μM. Ind, Mepac and AA were added to the bath 17 min before S₂ and PGE₂ was 2 min before S₂. The columns show the means with s.e. shown by vertical bars. Numbers of experiments are shown in parentheses. ***P* < 0.01, ****P* < 0.001 as compared with controls. **P* < 0.05 as compared with Ind. ^b*P* < 0.05, ^c*P* < 0.001 as compared with Mepac and AA.

Electrical field stimulation-induced release of acetylcholine

Previously, we showed that the EFS-induced release of ACh is not affected by Ind (Yagasaki *et al.*, 1984). In the present study, the influence of endogenous arachidonic acid on the release of ACh stimulated by EFS was examined. Mepacrine (5 μM) significantly reduced the EFS-induced release of ACh and this inhibition was overcome by 10 μM arachidonic acid. The effect of arachidonic acid was not altered by 2.8 μM Ind (Figure 4).

Discussion

Both DMPP and nicotine induce the release of ACh by stimulating the nicotinic receptors on the soma of neurones since this release is inhibited by hexamethonium and TTX (Vizi, 1973). Substance P also excites the soma of enteric neurones (Wood, 1987) and causes the TTX-sensitive release of ACh

Figure 3 Effects of indomethacin (Ind), mepacrine (Mepac) and arachidonic acid (AA) on 5-hydroxytryptamine (5-HT)-induced release of acetylcholine (ACh) from the myenteric plexus of guinea-pig ileum. 5-HT was present at 2.6 μM. Mepac, AA and Ind were added to the bath 17 min before S₂. The columns show the means with s.e. shown by vertical lines. Numbers of experiments are shown in parentheses. **P* < 0.05, ***P* < 0.01 as compared with controls. **P* < 0.05, as compared with Mepac and AA.

Figure 4 Effects of mepacrine (Mepac) and arachidonic acid (AA) on electrical field stimulation (EFS)-induced release of acetylcholine (ACh) from the myenteric plexus of guinea-pig ileum and the effect of indomethacin (Ind) on the recovery caused by AA. EFS was applied at supramaximal voltage (50 V), 0.5 ms duration and 10 Hz for 20 s. Mepac, AA and Ind were added to the bath 17 min before S₂. The columns show the means with s.e. shown by vertical bars. Numbers of experiments are shown in parentheses. **P* < 0.05, ***P* < 0.01 as compared with controls. **P* < 0.05, as compared with Mepac and AA.

(Yau & Youter, 1982). In the present study, Ind (2.8 μM) inhibited the release of ACh induced by DMPP and substance P. The concentration of Ind used in this experiment causes a decrease in the production of endogenous prostaglandins by inhibiting the activity of cyclo-oxygenase in this tissue (Flower & Vane, 1974; Botting & Salzman, 1974). In addition to inhibiting prostaglandin synthesis, Ind has various actions (Northover, 1977), but these effects occur at concentrations more than 10 times higher than that used in the present experiments. Thus, the inhibitory effects of Ind on the release of ACh by these substances may be attributed to decreases in levels of prostaglandins. This hypothesis is supported by the evidence that the addition of PGE₂ overcame the Ind-induced inhibition of the release of ACh evoked by DMPP and substance P. In addition, it has been reported that Ind depresses the release of ACh that is induced by nicotine (Yagasaki *et al.*, 1984), by bradykinin (Yau *et al.*, 1986) and during the atropine-sensitive contraction that is evoked by cholecystokinin (Zsely *et al.*, 1979); the inhibition in each case was overcome by the addition of PGE. These results suggest that endogenous prostaglandins contribute to the maintenance of the excitability of cholinergic neurones by substances that activate the receptors on the soma of myenteric neurones of the guinea-pig ileum. Previously, we showed that the release of ACh induced by the stimulation of axons or terminals was not affected by 2.8 μM Ind (Takeuchi *et al.*, 1989). Thus, the decrease in production of prostaglandins may affect neither the excitability of the axons nor of the terminals of cholinergic neurones.

In contrast to the above results, the release of ACh induced by 5-HT was not inhibited by Ind, although 5-HT has been reported to induce the release of ACh stimulating the 5-HT receptors on the enteric neurones (Taylor & Bywater, 1989). The reason why Ind was ineffective on the release of ACh evoked by 5-HT is not clear at present, but in contrast to the experiments with DMPP and substance P, about 40% of the release of ACh evoked by 5-HT still remained in the presence of TTX. Furthermore, it has been shown that the neuronally-mediated contraction of guinea-pig ileum to 5-HT was resistant to hexamethonium (Sanger & Nelson, 1989), and that the application of 5-HT to the soma of myenteric neurones did not affect the membrane potential in most neurones (Galligan *et al.*, 1988). In our model, therefore, the action of 5-HT may be on the axons and/or terminals. This site of action is consistent with the inability of Ind to affect the release of ACh

induced by the stimulation of axons and terminals (see earlier) and with the evidence that prostaglandins have no effects on the excitability of axons and terminals (Yagasaki *et al.*, 1984).

Mepacrine inhibited the release of ACh induced by all the stimuli used and such inhibition was at least partly overcome by the addition of arachidonic acid. Mepacrine inhibits the activity of phospholipase A₂ (Blackwell *et al.*, 1981) which plays an important role in the release of neurotransmitters (Takeuchi & Yagasaki, 1987). Thus, the inhibitory effect of mepacrine on the release of ACh in the present experiments may be due to the depression of phospholipase A₂ activity. The inhibition of phospholipase A₂ activity would cause a decrease in the liberation of arachidonic acid from phospholipids in membranes and, in turn, it would lower the level of prostaglandins in the tissue (Irvine, 1982; Needleman *et al.*, 1986). As mentioned above, prostaglandins modulated the release of ACh induced by DMPP and substance P. Since Ind cancelled the restorative effects of arachidonic acid on the release of ACh induced by DMPP and substance P, the effect of arachidonic acid may not be attributable to arachidonic acid itself but to its metabolites, the prostaglandins. However, we showed previously that the inhibition by mepacrine was not overcome by PGE₂ alone, and that arachidonic acid is also needed (Takeuchi & Yagasaki, 1987). Thus, these results suggest that the release of ACh induced by DMPP and substance P may also be regulated by both arachidonic acid and prostaglandins.

Ind did not inhibit the restorative effect of arachidonic acid on the EFS-induced release of ACh in the presence of mepacrine. This result is consistent with the result of Yagasaki *et al.* (1984) who found that prostaglandins are not involved in the

modulation of the EFS-induced release of ACh. It is known that arachidonic acid is metabolized to lipoxygenase products as well as cyclo-oxygenase products. In the guinea-pig ileum, however, the lipoxygenase products can be considered not to participate in the effect of arachidonic acid on the release of ACh (Takeuchi & Yagasaki, 1987). Thus, arachidonic acid itself may regulate the release of ACh that is stimulated by EFS. EFS excites the axons in the neurones of the myenteric plexus (Yagasaki *et al.*, 1984) and the invasion of the propagated action potential to nerve terminal induces the release of ACh (Paton *et al.*, 1971). Thus, it appears that arachidonic acid may contribute to the modulation of the axonal conductance of neurones or of some aspect of the mechanism of the release of transmitter in the nerve terminals. Arachidonic acid can stimulate the release of hormones by raising intracellular concentrations of Ca²⁺ (Kolesnick *et al.*, 1984) or by activating the turnover of phosphatidylinositol (Zeitler & Handwerker, 1985). In the release of neurotransmitter, Ca²⁺ and the turnover of phosphatidylinositol play important roles (Gershon, 1981; Nahorski *et al.*, 1986). From these findings, it seems conceivable that in the process involved in the release of ACh within the nerve terminals, there is a step that is sensitive to arachidonic acid.

In conclusion, endogenous prostaglandins may contribute to the modulation of excitability of the myenteric plexus by DMPP and substance P but not by 5-HT. In addition, endogenous arachidonic acid, a precursor of prostaglandins, also plays an important role in the release of ACh from nerve terminals of cholinergic neurones in the myenteric plexus of the guinea-pig ileum.

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(Received May 15, 1990)

Revised July 30, 1990

Accepted October 8, 1990)