A Rational Superfusion Medium for the Bioassay of E-Type Prostaglandins on the Rat Stomach Strip

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The optimum composition of a mixture of antagonist to be used in the bioassay of E-type prostaglandins was determined for the rat stomach strip (RSS). In the presence of atropine (10^{-7}M), indomethacin (10^{-6}M), propranolol (10^{-4}M), and tolazoline (10^{-4}M) the sensitivity of the RSS to muscarinic, α and β adrenergic and serotonergic agonists was greatly reduced whereas its responsiveness to PGE_1 and PGE_2 was unaltered. Using the oil-immersion-superfusion technique with this drug mixture, the bioassay of prostaglandins from samples also containing other agonists gave accurate estimates of the PG concentration of the samples using small amounts of 10^{-6}M PGE_1 or PGE_2.

Key Words: PG-bioassay; Rat stomach strip; pA_{10}; Prostaglandins; Antagonist mixture; Indomethacin

INTRODUCTION

The pharmacologist's own technique of the bioassay (Gaddum, 1964) is still favoured for the determination of prostaglandins (PGs) in biological extracts. However, the indicator tissues used have generally been introduced to assay other agents than PGs (Vane, 1957). Therefore, a wide variety of drugs and dosages are currently used in combination to make the preparation selectively sensitive to PGs. Surprisingly, arguments for their composition are generally lacking (Orehek et al., 1975; Gilmore et al., 1968), even with the introduction of new indicator tissues (Vapaatalo et al., 1976). To obtain a rational mixture of antagonists we used the following guidelines:

Use an antagonist only if the relevant pD_2 of the agonist for the tissue exceeds three.

Use low effective antagonist concentrations; for this purpose the pA_{10} was chosen.

If possible, use antagonists with affinities for more than one receptor (Schechter and Weinstock, 1974).

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To test these guidelines, the bioassay of PGEs with the rat stomach strip (RSS) was chosen.

METHODS

Fed, male Wistar rats (CPB-TNO, Zeist, The Netherlands) weighing about 200–300 g were killed by decapitation. After opening the abdomen, the gastric rumen (often misnamed fundus) (Pernkopf, 1930) is dissected by cutting the esophagus and the stomach along the cardiac ridge, rinsed with Krebs solution and cut in a strip, made by cutting a longitudinal strip, 2–3 mm wide, from the rumen along the greater curvature parallel to the longitudinal muscle, 2–4 cm long.

The composition of the Krebs solution used was (mM): NaCl(95), KCl(4.7), $MgSO_4$(1.2), $CaCl_2$(2.5), KH_2PO_4(1.2), $NaHCO_3$(25) and glucose (11.6). It was warmed to 37°C and gassed with 95% O_2, 5% CO_2; the pH was 7.4.

To determine the affinities of agonists and antagonists the strips were mounted in an organ-bath filled with 10 ml of Krebs solution and contractions were measured isotonically against a load of 8 mN with a displacement transducer (Hottinger-Baldwin W_{10} and MC 1A amplifier) using a vibrator to overcome friction. Cumulative dose-response curves were made with two steps per decade throughout the concentration range used. The error made by dilution was maximally 2% per decade and generally less than 5% per experiment. Responses to relaxing agents were measured on strips contracted by and in the presence of 3×10^{-7}M carbachol. A two minute equilibration was used for the antagonists to determine their affinity.

PGs were bioassayed with the oil-immersion technique (Ferreira and De Souza Costa, 1976) using the same apparatus for measuring contractile responses. The preload used in this kind of experiments was 20 mN. The strips were superfused at a rate of 0.2 ml/min with Krebs solution. Strips were equilibrated by superfusion with the Krebs solution containing indomethacin with or without antagonists for at least one-half hour before the assay started. An aliquot of 25 μl of the solution assayed was added to the superfusion fluid 5 mm above the strip. The interval between each addition was 10 minutes, during which the strip spontaneously relaxed to its baseline.

In both types of experiments, the RSS was allowed to reach a steady-state baseline for about one hour after mounting, while it was superfused continually or washed repeatedly.

The pD_2 values, used as a measure of agonist affinities, were calculated from the dose-response curves as the mean (± SEM) of estimates of the concentration inducing a half-maximal response. The pA_2 and pA_{10} values, which were calculated with their confidence limits (95%) from Arunlakshana-Schild plots (AS plots) (Arunlakshana and Schild, 1959), were made with at least three different antagonist concentrations and at least three independent measurements at each concentration. Plots were assumed to be valid when there was no significant deviation from linearity and when the slope was significantly different from zero at the 5% level (Diem and Lentner, 1971). The assumption of competitive antagonism was tested

by determining the deviation of the regression from unity. Probability values at or below 5% were considered statistically significant.

Drugs used were: acetylcholine chloride (OPG), adrenaline bitartrate (OPG), arachidonic acid (Merck), atropine sulfate (Merck), carbachol (OPG), cimetidine (SK & F), histamine di-HCl (Merck), hyoscine N-methyl-bromide (Merck), indomethacin (MSD), isoprenaline (N-isopropyl-dl-noradrenaline hydrochloride, Fluka), metaraminol bitartrate (MSD), 4-methyl-histamine-di-HCl (SK & F), methysergide HML (Sandoz), dl-muscarine chloride (Sigma), liquid paraffin (viscosity: 26–35 cP, OPG), phentolamine HCl (Ciba-Geigy), propranolol HCl (ICI), prostaglandin E_1, E_2 (Unilever), pyrilamine HCl (Specia), serotonin-creatinin sulphate (Merck), tolazoline HCl (Ciba-Geigy). The concentrations given refer to the active principle.

RESULTS

Agonists

The pD_2 values of the agonists studied are given in Table 1; the tissue sensitivities are similar to those reported (Vane, 1957). Strips hardly contracted in the presence of histamine up to 10^{-2}M, but contracted vigorously when the drug was washed out. Therefore, approximate pD_2 values of histamine were measured with the oil-immersion-superfusion technique, taking the histamine concentration in the addition as agonist concentration. The pD_2 values thus measured indicate that the tissue is rather insensitive to histamine. Similar results were found using methylhistamine and metiamide. Neither a contraction via H_1 receptors nor a relaxation via H_2 receptors is likely to interfere with the bioassay of PGs on the RSS.

TABLE 1 Sensitivity of the Rat Stomach Strip to Several Agonists

AGONIST	$pD_2 \pm$ SEM	*N*
Acetylcholine	5.99 ± 0.12	31
Muscarine	6.41 ± 0.29	6
Metaraminol	5.55 ± 0.07	11[a]
Adrenaline	6.96 ± 0.08	11[a]
Isoprenaline	7.02 ± 0.10	7[a]
Serotonin	7.38 ± 0.11	26
Histamine	>1	6[b,c]
Histamine	1.29 ± 0.13	4[a,b,d]
Prostaglandin E_1	7.14 ± 0.09	5

N = number of strips.

[a] Relaxing responses, measured with strips contracted by 3×10^{-7}M carbachol.

[b] Oil-immersion-superfusion technique.

[c] In the presence of 10^{-6}M cimetidine.

[d] In the presence of 5×10^{-6}M pyrilamine.

Antagonists

Table 2 summarizes the effects of several antagonists. The difference between the pA_2 and the pA_{10} values for atropine does not indicate a competitive antagonism between atropine and acetylcholine on the muscarinic receptor. The difference of the regression of the AS-plot (Fig. 2) in the first experiment from unity might be due to the use of two or three antagonist concentrations on one preparation (Fig. 1). Therefore, the experiment was repeated using one antagonist concentration per strip. As can be seen in Table 2, this experiment yielded similar results. The regression of both these experiments, 2.09 and 2.11 respectively, differed significantly from unity, the value expected with competitive antagonists. This apparently noncompetitive action of atropine might be due to nonspecificity, as assumed by Vane (1957) who therefore preferred hyoscine to the effects of acetylcholine mediated via nicotinic receptors. However, hyoscine and muscarine (Table 2) did not appear competitive either.

Mixture of Antagonists

Composition

With the data given in the preceding section and along the guidelines presented in the introduction, the following mixture of antagonists was made: atropine 10^{-7}M, propranolol 10^{-4}M, and tolazoline 10^{-4}M. These concentrations are rounded off pA_{10} values, but the concentration of propranolol used was ten times higher than needed for its β-adrenoceptor blocking activity, in order to make a specific antagonist of serotonin superfluous.

To inhibit the synthesis of PGs by the rat stomach strip, indomethacin (10^{-6}M) was added. This concentration, indicated by Grygelwski (1977), inhibited completely the contractile response of the oil-immersion-superfused strip to 25 μl of 10^{-4}M arachidonic acid solution.

TABLE 2 The pA_2 and pA_{10} Values of Antagonists. The pD_2 Values of the Agonist Concerned are also Given. Further Details, see Text.

ANTAGONIST	AGONIST	pA_2 (95% CL)	pA_{10} (95% CL)	N	pD_2 (± SEM)[b]
Atropine	Acetylcholine	8.02 (7.89–8.27)	7.56 (7.48–7.62)	7	6.87 ± 0.24
Atropine	Acetylcholine	7.82 (7.71–8.65)	7.40 (6.98–7.50)	15	5.69 ± 0.08
Atropine	Muscarine	7.70 (7.59–7.91)	7.15 (6.98–7.26)	6	6.41 ± 0.29
Hyoscine	Acetylcholine	8.18 (7.69–8.75)	7.73 (7.43–8.05)	8	5.76 ± 0.09
Methysergide	Serotonin	8.12[a]	6.79[a]	10	7.49 ± 0.17
Propranolol	Serotonin	5.55 (5.31–5.85)	4.53 (4.26–4.70)	9	7.12 ± 0.19
Propranolol	Isoprenaline	6.08 (5.80–7.74)	5.36 (4.95–5.47)	7	7.02 ± 0.10
Propranolol	Adrenaline	6.63[a]	5.16[a]	6	6.81 ± 0.07
Tolazoline	Adrenaline	4.64 (4.50–4.90)	3.48 (2.78–3.76)	5	7.13 ± 0.22
Tolazoline	Metaraminol	5.27 (4.94–6.10)	4.31 (4.18–4.46)	6	5.44 ± 0.08

N = number of strips.

[a] Data presented, although the AS-plot had a linear relationship without a regression significantly different from zero.

[b] These data are part of the result summarized in Table 1.

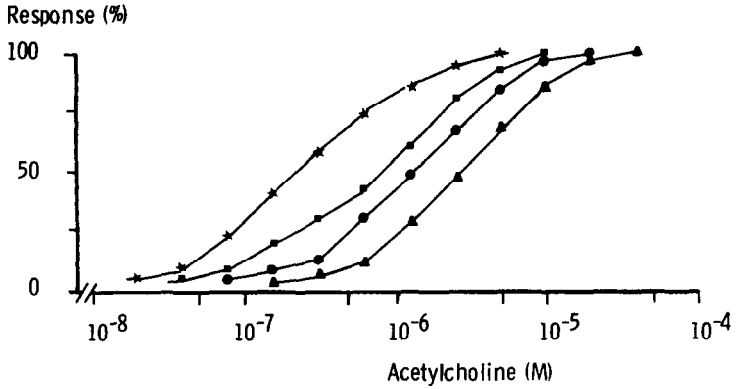

FIGURE 1. Dose–response curves of acetylcholine in the absence (★) and the presence of atropine (■ 1.6 × 10^{-8}M; ● 2 × 10^{-8}M; ▲ 3 × 10^{-8}M). Contractions of the rat stomach strip were recorded isotonically (preload = 8 mN; 100% = 16 mm). In order to determine more accurately the shape of the curves a smaller dose interval was used in this experiment.

Performance

To determine the effect of this antagonist mixture on the sensitivity of the rat stomach strip to PGs, dose-response curves of PGE_1 and PGE_2 were made with two separate strips from the same stomach superfused simultaneously with Krebs solution alone and with Krebs solution containing antagonists. The sensitivity to PGE_1 and PGE_2 was not significantly changed by the drug mixture (Table 3).

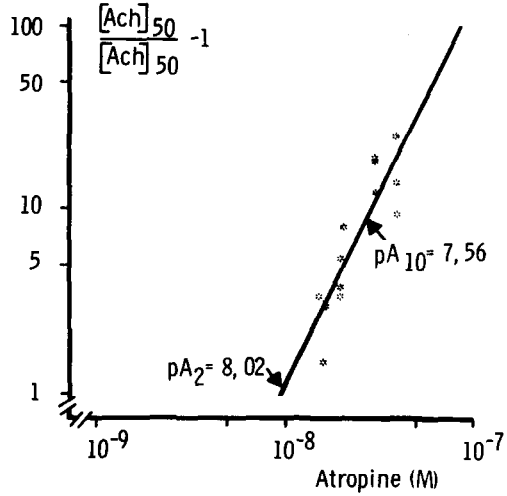

FIGURE 2. Arunlakshana-Schild plot of the antagonism between acetylcholine and atropine on the rat stomach strip. Statistical analysis: Linearity: p = 0.87; zero-regression: p = 0.0001; regression unity: p = 0.012; regression (± 95% cl) = 2.09 ± 0.81. For further details see Table 2.

TABLE 3 Sensitivity of Rat Stomach Strips to PGE_1 and PGE_2 in the Presence and in the Absence of the Antagonist Mixture

	Without antagonists		With antagonists	
	$pD_2 \pm$ SEM	N	$pD_2 \pm$ SEM	N
PGE_1	5.71 ± 0.10	6	5.92 ± 0.14	6
PGE_2	6.16 ± 0.08	8	6.03 ± 0.12	8

N = number of strips.

N.B., pD_2 values were estimated using the agonist concentration in the addition (25 μl) for the dose-response curves.

Likewise, in organ bath experiments the dose-response curves of PGE_2 (10^{-9} to 10^{-7}M, $n = 2$) made in the presence of the antagonists (equilibration = 30 min) were identical to those made on the same strip before the addition of antagonists. As a final test three different mixtures of PGs with other agonists were assayed with the oil-immersion-superfusion technique by a technician who did not know their composition. The results of the 2 + 2 point assays (*European Pharmacopoeia*, 1971) are presented in Table 4.

DISCUSSIONS

The results indicate that atropine 10^{-7}M, indomethacin 10^{-6}M, propranolol 10^{-4}M, and tolazoline 10^{-4}M render the RSS more selectively responsive to E-type prostaglandins, without diminishing its sensitivity. Comparison of the pD_2 values obtained in organ bath experiments with those observed with the oil-immersion-superfusion

TABLE 4 Two-plus-two Point Bioassay of Prostaglandins in Mixtures with Other Agonists

		Assay outcome (95% confidence limits)	
Sample content (M)		Concentration (M)	% of sample content
PGE_2	3×10^{-6}	3.87×10^{-6} PGE_2	129
Adrenaline	10^{-6}	($3.02\text{–}4.82 \times 10^{-6}$)	(101–161)
PGE_2	4×10^{-6}	4.41×10^{-6} PGE_2	110
Adrenaline	10^{-6}	($3.57\text{–}5.54 \times 10^{-6}$)	(89–138)
PGE_2	3×10^{-6}	3.87×10^{-6} PGE_2	129
Serotonin	10^{-7}	($3.25\text{–}4.63 \times 10^{-6}$)	(108–154)
Histamine	10^{-5}		
Acetylcholine	10^{-6}		
PGE_1	8.2×10^{-4}	6.15×10^{-4} PGE_1	75
Acetylcholine	10^{-6}	($3.85\text{–}9.36 \times 10^{-4}$)	(47–114)
Histamine	10^{-3}		
Serotonin	10^{-7}		

technique indicates that in the present circumstances the latter technique appears to have a dilution factor of about 25. As already stressed by Ferreira and De Souza Costa (1976) the main advantage of this technique is that minute quantities of prostaglandins are detectable, a half maximal response being evoked by 10^{-11} mole (10^{-13} g) PGE_2.

Although the threshold sensitivity of our method (10^{-14} g PGE_2) is fifty times higher than that reported by Ferreira and De Souza Costa (1976), the threshold sensitivity of the tissue is similar (Bagshaw, 1978). As already stated by Gaddum (1953), the response of the superfused strip is proportional to the agonist concentration. Because it is unlikely that the receptor concentration of the tissue can increase, the apparently paradoxical situation occurs that by changing experimental circumstances, increased sensitivities of the bioassay (expressed in mole or g) are due to decreased volumes of assay solutions having the same or higher agonist concentrations (M or g/l). Thus, depending on the amount and on the expected PG concentration of the solution to be assayed, an optimal bioassay method, with regard to its dilution factor and its sensitivity has to be chosen.

From the results presented it can be concluded that the guidelines used in composing an antagonist mixture lead to optimal conditions for the bioassay of PGEs with RSS. It seems likely that this approach may be useful for other indicator tissues and/or other types of PG (cf Bult et al., 1977).

Only tentative explanations can be given for the apparent noncompetitive antagonistic action of atropine. Arunlakshana and Schild (1959) already reported this effect with atropine. They observed a competitive antagonism of acetylcholine by atropine on the guinea pig ileum only in the presence of hexamethonium after stabilizing the preparation by administering about 100 doses of acetylcholine. Furthermore, they apparently prolonged the equilibration time for atropine, but they did not report its duration. From our experiments with muscarine, it is clear that this effect cannot be due to stimulation of nicotinic receptors on ganglia in Auerbach's plexus; if indeed any are present in this esophageal-type of tissue. Also the paradoxical potentiating effects of low doses of atropine (10^{-9}M and lower) on contractions induced by acetylcholine are unlikely to have played a part in these experiments as the concentrations of atropine used (2×10^{-8}M and higher) were much higher (Arunlakshana and Schild, 1959). Another explanation might be that the AS-plot is not applicable because the assumption that the fraction of atropine bound to receptors can be ignored is not valid within the concentration range used. However, Arunlakshana and Schild (1959) reported that when inhibition was competitive, only a higher pA_2 value was observed and that the pA_{10} value remained similar. Therefore, it is likely that the pA_{10} values reported and used in the composition of the antagonist mixture do not differ from those obtainable in circumstances producing a competitive antagonism between acetylcholine and atropine or hyoscine.

Recently Manku et al. (1977) reported that propranolol may not be a suitable drug for use in PG bioassay systems; especially because the inhibition of RSS responses to low PG concentrations by propranolol would affect the bioassay. However, careful examination of dose-response curves of PGE_2 made before and

30 minutes after changing to the mixture of antagonists did not suggest effects similar to those reported (Manku et al., 1977), although comparable concentrations of propranolol were used. An explanation might be found in the fact that in our experiments strips were routinely washed every 5 minutes with Krebs solution containing antagonists, whereas Manku et al. reported that they left the strip in buffer containing propranolol for 20 minutes, while the control curves were made after repeated washing. Thus, it seems possible that the RSS reversibly deteriorates during a prolonged stay in the same buffer solution, as other explanations such as different strains of rats, different buffer solutions, or temperature difference are even more unlikely.

The present observation, that histamine induces contractions only in high concentrations and relaxations in lower concentrations, confirms earlier reports qualitatively (Vane, 1957; Ercan and Türker, 1977). However, taking into account the dilution factor, a pD_2 value of about 2.7 can be estimated from our experiments; whereas values as high as 7.3 were reported (Ercan and Türker, 1977). As we were unable to produce similar results either by changing experimental conditions or by using methyl-histamine and metiamide instead of histamine and cimetidine respectively, no plausible explanation of the quantitative difference between our results and those of Ercan and Türker (1977) can be given. It is possible that their data do not refer to concentrations (M) but to quantities (mole) of agonists, because they used a superfusion technique and indicated quantities in the recordings shown (Fig. 2), while using the same values as concentrations in the dose-response curve derived from it (Fig. 1). Furthermore, when the results presented in this paper are expressed as moles instead of moles per litre, they are identical with those reported (Ercan and Türker, 1977).

Although optimal conditions for the bioassay of prostaglandins can be found, the possibility always remains that even after extraction the samples to be assayed also contain active principles for which no antagonists are known (Ally et al., 1977). As more selective antagonists of prostaglandins become available, their effects on the responses of the indicator tissue induced by the samples might remove this uncertainty in the bioassay.

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