Br. J. Pharmac. (1983), **78**, 339–351

Distinctive pharmacological profile of a nonadrenergic inhibitory system in bullfrog lung

Hall Downes & Stephen M. Taylor[1]

Department of Pharmacology, School of Medicine, Oregon Health Sciences University, Portland, Oregon, 97201, U.S.A.

1 Bullfrog hemilungs, pretreated with atropine, are markedly relaxed on addition of carbachol. Since the relaxant effect is inhibited by tetrodotoxin or hexamethonium, it is neurally mediated and involves stimulation of nicotinic receptors with release of an unknown inhibitory transmitter.

2 Carbachol-induced relaxation is nonadrenergic since: (a) it considerably exceeds the maximal effects of isoprenaline or the effect of 10^{-3}M adrenaline or noradrenaline; (b) it elicits marked further relaxation in preparations already relaxed by high concentrations of catecholamines; (c) it is not attenuated by low concentrations of propranolol (10^{-6} and 3×10^{-6}M) that competitively antagonize isoprenaline-induced relaxation.

3 Carbachol-induced relaxation has multiple distinguishing characteristics, which serve as a fingerprint for the unknown inhibitory transmitter. These include an exceptionally rapid onset of action, a ceiling effect at 50% of maximal relaxation, and minimal retardation by concentrations of procaine that block or markedly retard relaxant responses to all other agonists.

4 This distinctive pharmacological profile cannot be reproduced by addition of exogenous catecholamines, 5-hydroxytryptamine, adenosine triphosphate (ATP) or adenosine, or by addition of ATP or adenosine following pretreatment with indomethacin. Furthermore, addition of carbachol to preparations previously relaxed with 10^{-3}M concentrations of these agents produced marked, additional relaxation.

5 Maximally effective concentrations of vasoactive intestinal peptide produced a barely detectable relaxant response equivalent to 8% of maximal relaxation. The response was totally prevented by pretreatment with procaine.

6 Carbachol-induced relaxation was not impaired by pretreatment with 10^{-4}M indomethacin.

7 Carbachol-induced relaxation of bullfrog lung therefore involves a postganglionic inhibitory transmitter that in nonadrenergic, non-5-hydroxytryptaminergic, and nonpurinergic, and whose effects are not dependent on prostaglandin synthesis. Although a peptide may function as the inhibitory transmitter, it is not vasoactive intestinal peptide.

Introduction

A nonadrenergic, noncholinergic inhibitory system innervating lung, gut and other viscera has been demonstrated in all vertebrate classes (Burnstock, 1969; 1972), and is believed to be the major inhibitory innervation to anuran lung (Burnstock, 1969). Ultrastructural studies (Robinson, McLean & Burnstock, 1971; Campbell, Haller & Rogers, 1978) show that about 50% of the efferent terminals in toad lung contain the distinctive large opaque vesicles regarded as characteristic of the nonadrenergic, noncholinergic inhibitory system.

Previous studies in toad (*Bufo marinus*) lung have shown that the inhibitory innervation accompanies the vagus nerve (Wood & Burnstock, 1967; Campbell, 1971), and that the inhibitory effects of vagal stimulation can be prevented by ganglionic blockade (Wood & Burnstock, 1967; Campbell & Duxson, 1978), but not by atropine (Wood & Burnstock, 1967; Campbell, 1971). Similar inhibitory effects have been elicited in toad (*B. marinus*) and frog (*Rana esculenta*) lungs with ganglionic stimulants

[1]Present address: Pulmonary Research Laboratory, St. Paul's Hospital, 1081 Burrard Street, Vancouver, BC V62 1Y6, Canada.

0007-1188/83/020339-13 $01.00

(Wood & Burnstock, 1967; Schnizer, Hoang & Brecht, 1968) such as dimethylphenylpiperazinium (DMPP). Although the response is easily elicited, very little is known about the inhibitory transmitter or transmitters involved.

Exogenously administered adrenaline (Carlson & Luckhardt, 1920; Brecht & Fraessle, 1944; Meves, 1953; Kobayasi & Yoda, 1960; Wood & Burnstock, 1967, Holmgren & Campbell, 1978), 5-hydroxytryptamine (Schnizer *et al.*, 1968) and adenosine triphosphate (ATP) (Meves, 1953) produce some degree of relaxation of anuran lung, and there is evidence from previous studies supporting each of these agents as a putative transmitter for neurally mediated inhibition. Vasoactive intestinal peptide, which has been proposed as the transmitter for nonadrenergic, noncholinergic inhibition in mammalian airways (Matsuzaki, Hamaski & Said, 1980) has not been studied in anurans. Adrenaline and 5-hydroxytryptamine have been identified in frog lung (*R. esculenta*) in relatively high concentrations (0.2 to 0.9 μg/g) and are released into perfusates during vagal stimulation (Schnizer *et al.*, 1968). Both β-adrenoceptor antagonists (Wood & Burnstock, 1967; Schnizer *et al.*, 1968) and drugs with anti-5-hydroxytryptamine actions (Schnizer *et al.*, 1968) have depressed neurally mediated relaxation of anuran lung. However, since the concentrations of β-adrenoceptor antagonists used (approximately 10^{-4}M), were considerably above those that produce selective effects on β-receptors, the observed antagonism could have resulted from depressant effects on ganglionic transmission or inhibitory transmitter release. These same considerations also apply to the effects of 5-hydroxytryptamine antagonists, which included drugs such as chlorpromazine and promethazine. Finally, although Burnstock (1972) has proposed that ATP is stored in the large opaque vesicles of the nonadrenergic, noncholinergic inhibitory system, the composition of these vesicles has not been determined, and the relaxant effects of ATP have not been compared with neurally mediated inhibition in anuran lung.

In order to assess the importance of a particular autacoid in the overall inhibitory response, it is necessary to establish both that it is present and released during inhibition, and that its pattern of pharmacological effects accurately reproduces the effects of the nonadrenergic, noncholinergic inhibitory system. The present study is concerned with the latter aspect, and employs nicotinic agonists in isolated preparations of bullfrog (*R. catesbeiana*) lung to elicit a highly consistent, postganglionic inhibitory response with a distinctive pharmacological profile. This profile cannot be reproduced by catecholamines, 5-hydroxytryptamine, ATP, adenosine or vasoactive intestinal peptide.

Methods

The lungs were removed from pithed *Rana catesbeiana* (weighing 150–350 g) and split longitudinally to provide four hemilungs. These were mounted vertically in jacketed 50 ml baths filled with a solution of the following composition (mM): NaCl 89, $NaHCO_3$ 20, KH_2PO_4 2.5, $CaCl_2$ 2.0, $MgSO_4$ 1.5 and glucose 10. The ionic composition is close to that reported for frog plasma (Fenn, 1936; Conway, 1957). To reduce spontaneous activity, the solution was maintained at 15°C with a Lauda K-2/R circulator and thermoregulator. The solution was aerated with a mixture of 1.3% CO_2 and 98.7% O_2 to give a pH of 7.8 (Downes & Taylor 1982), approximating the physiological values of CO_2 tension and blood pH for intact, unanaesthetized bullfrogs at 15°C (Howell, Baumgardner, Bondi & Rahn, 1970).

Changes in muscle length were recorded with isotonic transducers (Harvard 356) under a 2 g tension, and at a usual gain of 1×. Higher gains were employed in some experiments with catecholamines in order to measure their relatively slight inhibitory effect. The hemilungs were equilibrated for 2 to 3 h, until a stable resting length had been achieved, before testing drug effects. At the end of the experiment, the hemilungs were maximally relaxed by incubation overnight with 10^{-2}M theophylline; preceding drug effects were expressed as a percentage of this maximal effect (I_{Max}). Results from different hemilungs from the same frog ('matched' hemilungs) were compared by the *t* test for paired observations.

Drugs used were adenosine (Sigma), adenosine 5'-triphosphate disodium salt (ATP; Sigma), (−)-adrenaline bitartrate (Sigma), atropine sulphate (Sigma), carbamylcholine chloride (carbachol; Sigma), dipyridamole (Boehringer Ingelheim), 1-1-dimethyl-4-phenylpiperazinium (DMPP; Sigma), hexamethonium bromide (Sigma), 5-hydroxytryptamine HCl (Sigma), indomethacin (Sigma), (±)-isoprenaline HCl (Sigma), methysergide maleate (Sandoz), (−)-noradrenaline HCl (Sigma), procaine HCl (Sigma), propranolol HCl (Ayerst), quinidine sulphate (Sigma), tetrodotoxin (Sigma), theophylline (Sigma), and vasoactive intestinal peptide (MW 3326; Peninsula Laboratories). Indomethacin was dissolved in absolute ethanol and 125 μl of the ethanolic solution was added to a 50 ml bath to achieve indomethacin and ethanol concentrations of 10^{-4}M and 4.3×10^{-2}M, respectively; an equivalent concentration of ethanol was added to the matched hemilungs used as controls for indomethacin experiments. All other drugs were dissolved in distilled water and appropriately diluted with bath solution before addition to the baths. Dipyridamole was dissolved in a weakly acidic solution. Catecholamines were prepared as 10^{-1} or^{-2}M solu-

tions in water with 10^{-2}M ascorbic acid. Ascorbic acid was added to the baths in experiments employing catecholamines for a final concentration of 10^{-4}M. Unless the experiment was specifically testing the muscarinic effects of carbachol or the β-adrenoceptor effects of catecholamines, 10^{-5}M atropine and 10^{-6}M propranolol were added to the bath solution during the incubation period to prevent contraction on the subsequent addition of carbachol, and to prevent the possible inhibitory effects of endogenously released catecholamines, respectively. Atropine and propranolol had no effect on resting length. Other drugs used as potential antagonists of carbachol or autacoid effects were added to the bath solution at times indicated in the appropriate figure legends.

Results

Intrinsic tone

Bullfrog hemilungs slowly contracted during the first 2 to 3 h of incubation and thereafter maintained a stable resting length in the range of 1.5 to 2.5 cm. As previously reported, (Downes & Taylor, 1982), procaine has a paradoxical stimulant effect on bullfrog lung, and at 10^{-4}M concentration elicits maximal contraction. In the present experiments procaine produced only 0.34 ± 0.13 cm (mean ± s.e., $n = 13$) of contraction, indicating that the hemilungs were almost fully contracted in the resting state. Subsequent addition of 10^{-2}M theophylline to the same preparations caused 3.58 ± 0.24 cm of relaxation from the maximally contracted state.

Carbachol-induced relaxation

In the absence of other drugs, carbachol caused dose-related contraction from 10^{-8} to 10^{-5}M, and a variable degree of relaxation as the concentration was increased to 3×10^{-5} or 10^{-4}M. Hemilungs were routinely pretreated with 10^{-5}M atropine to prevent contractile responses to the lower concentrations of carbachol. Under these conditions, carbachol produced a threshold relaxant effect at 10^{-5}M, and marked relaxation at 3×10^{-5}M (Figure 1a). Increasing the concentration to 10^{-4}M elicited the maximal relaxant response to carbachol, which was frequently of a biphasic nature (Figure 1a); only the initial relaxation was employed for measurement of dose-response relationships at this concentration. Increasing the concentration above 10^{-4}M elicited a variable degree of contraction (Figure 1a). The maximal relaxant response to carbachol was equivalent to about half of I_{max} and showed no evidence of seasonal

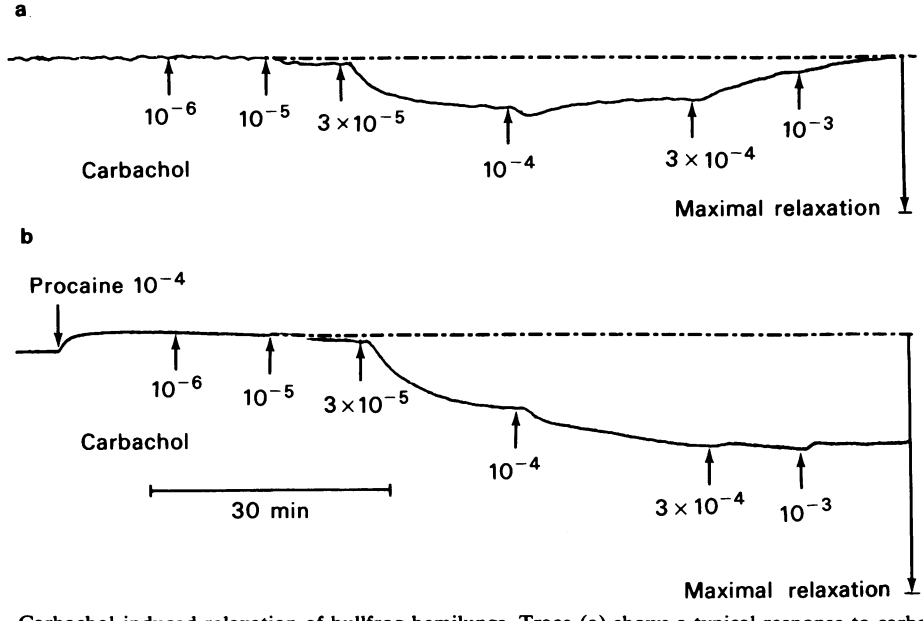

Figure 1 Carbachol-induced relaxation of bullfrog hemilungs. Trace (a) shows a typical response to carbachol. Trace (b) shows the carbachol response in a matched hemilung which has been maximally contracted with procaine. Propranolol (10^{-6}M) and atropine (10^{-5}M) were added to the baths in the initial equilibration period, about 60 min before testing carbachol. Addition of drug is indicated by an arrow with the cumulative drug concentration (M) appended. Large vertical arrows to the right show I_{max}, as obtained after subsequent relaxation with theophylline (10^{-2}M). The dashed line indicates length (baseline) before addition of carbachol.

Table 1 Lack of seasonal variation for carbachol-induced relaxation*

	Sept–Oct ($n = 10$)	*Jan–Feb* ($n = 6$)	*April–May* ($n = 16$)	*July–Aug* ($n = 15$)
Carbachol 10^{-4}M, % of I_{max}†	49 ± 5 (33–75)	50 ± 5 (34–63)	51 ± 4 (26–68)	52 ± 5 (36–77)
I_{max}, mm of relaxation†	34 ± 2 (28–40)	30 ± 1 (28–34)	30 ± 2 (21–42)	30 ± 2 (15–36)

*Hemilungs pretreated with atropine 10^{-5}M and propranolol 10^{-6}M.
†Mean ± s.e. (range).

variation (Table 1). Repeated administration did not produce tachyphylaxis (Table 2). However, the extent of the contractile response to high concentrations of carbachol ($> 10^{-4}$M) showed considerable variation between animals, and occasional batches of frogs demonstrated little or no contraction (Figure 2b and f).

Effects of atropine, hexamethonium, tetrodotoxin, quinidine and dipyridamole on carbachol-induced relaxation

Varying the concentration of atropine from 10^{-7} to 3×10^{-5}M (Figure 2a) did not alter either carbachol-induced relaxation or the contractile response to carbachol concentrations above 10^{-4}M. Hexamethonium surmountably antagonized both carbachol-induced relaxation (Figure 2b), and the virtually identical relaxation elicited by DMPP (Figure 3). Tetrodotoxin (Figure 2c) and quinidine (Figure 2d) produced an insurmountable block of carbachol-induced relaxation. Dipyridamole (Figure 2f) had no effect on carbachol-induced relaxation.

Procaine resistance

In hemilungs contracted with a subanaesthetic concentration of procaine, 10^{-4}M, the relaxant effects of most inhibitory agonists were greatly retarded or blocked (Figure 4). Carbachol-induced relaxation had a slightly slower onset in procaine-treated hemilungs, but by 15 min after addition, the response to 10^{-4}M carbachol was not significantly different in control hemilungs and hemilungs contracted with procaine (Figure 4). At this time, the relaxant responses to theophylline, isoprenaline, 5-hydroxytryptamine and vasoactive intestinal peptide were barely detectable or absent in the procaine-treated preparations. In other experiments ($n = 3$), increasing the concentration of procaine to 10^{-3}M, a local anaesthetic level (Dohadwalla, Freedberg & Vaughan Williams, 1969), resulted in a blockade of carbachol-induced relaxation.

Procaine, 10^{-4}M, had no effect on the dose-response relationships for carbachol-induced relaxation (Figure 2e), but prevented the contractile response to carbachol concentrations above 10^{-4}M (Figure 1b and 2e), demonstrating that these two effects of carbachol could be separated by pharmacological means. Despite the block of the high dose contractile response, increasing the carbachol concentration above 10^{-4}M (Figure 1b and 2e) produced little or no further relaxation.

Comparison with catecholamines and 5-hydroxytryptamine

Relaxant responses to catecholamines and 5-hydroxytryptamine required high drug concentrations, were of relatively small magnitude, and were highly variable from preparation to preparation. Adrenaline ($n = 7$) and noradrenaline ($n = 5$) caused dose-related relaxation from 10^{-6}M to 10^{-3}M, producing peak effects at the highest concentration which were equivalent to 14 ± 2 and 19 ± 2% of I_{max}, respectively. Isoprenaline elicited a clear maximal effect at 10^{-5} or 10^{-4}M concentration (Figure 5a). Relaxation elicited by isoprenaline averaged

Table 2 Relaxant responses* to five successive† administrations of 3×10^{-5}M carbachol

1st dose	*2nd dose*	*3rd dose*	*4th dose*	*5th dose*
45 ± 6	44 ± 8	43 ± 8	47 ± 8	46 ± 7

* % of I_{max}, mean ± s.e. ($n = 4$)
†Preparations were incubated with carbachol for 1 h at each exposure, and then washed and allowed to return to resting length before addition of next dose.

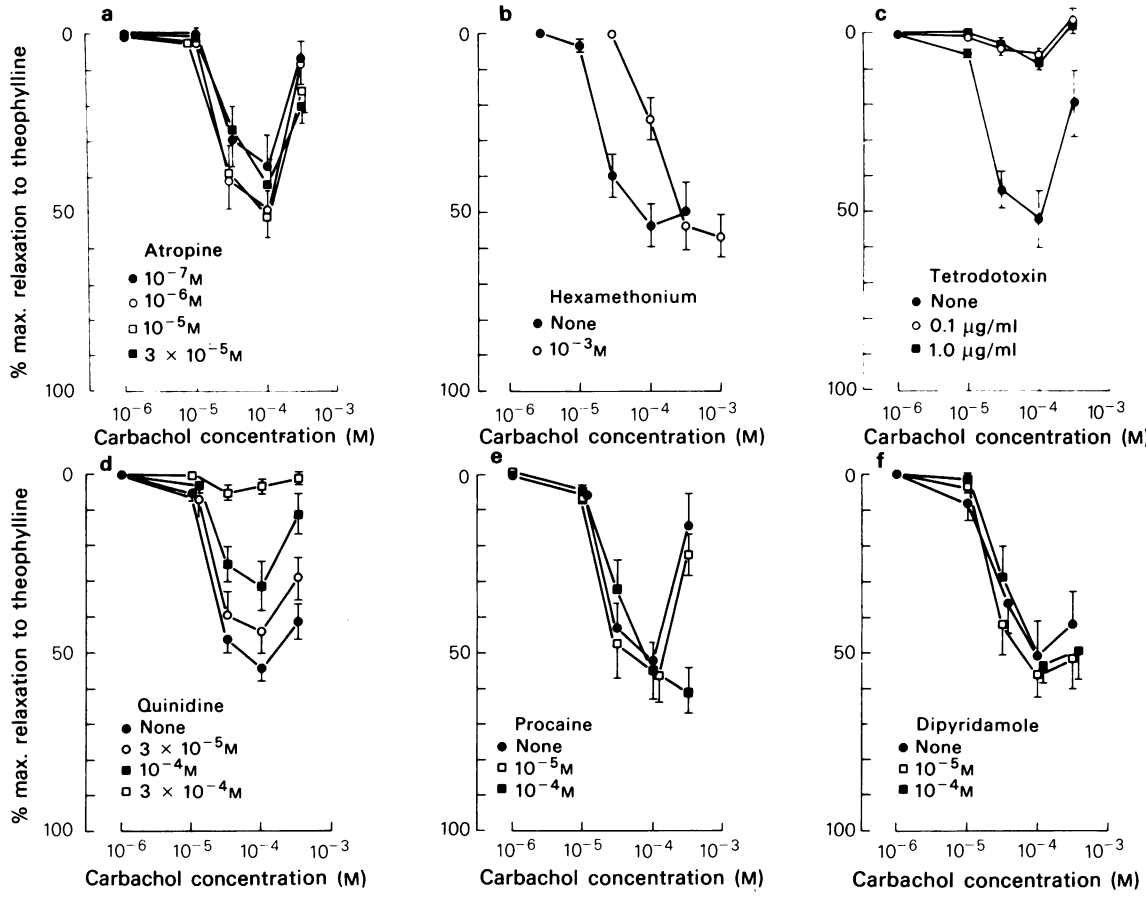

Figure 2 Cumulative concentration-effect curves for carbachol-induced relaxation of bullfrog hemilungs. (a) Lack of effect of varying concentrations of atropine; (b) antagonism by hexamethonium; (c) antagonism by tetrodotoxin; (d) antagonism by quinidine; (e) procaine spares relaxant effect; (f) lack of potentiation by dipyridamole. Responses are expressed as % of the maximal relaxation (I_{max}) subsequently elicited by theophylline. Propranolol (10^{-6}M) and atropine (10^{-5}M) were added during the initial equilibration period for series (b) to (f). Series (a) was not pretreated with propranolol. Atropine (a) and dipyridamole (f) were added to the baths at least 45 min before carbachol; hexamethonium (b) and quinidine (d) at 30 min before carbachol; tetrodotoxin (c) and procaine (e) at 15 min before carbachol. Each series represents matched hemilungs from the same animals. Means with s.e. (a) and (b), $n = 5$. (c) to (f), $n = 4$.

$21 \pm 4\%$ of I_{max} ($n = 9$; Figures 4 and 5). Propranolol surmountably antagonized isoprenaline-induced relaxation (Figure 5a). In control hemilungs ($n = 5$), 10^{-6}M isoprenaline elicited $44 \pm 8\%$ of the maximal effect of isoprenaline; in matched hemilungs pretreated with 10^{-6}, 3×10^{-6} and 10^{-5}M propranolol, the response to this concentration of isoprenaline was reduced to 20 ± 7 ($P < 0.05$), 14 ± 5 ($P < 0.05$) and 6 ± 2 ($P < 0.01$) % of the isoprenaline maximum. The lower concentrations of propranolol (10^{-6} and 3×10^{-6}M) had no effect on carbachol-induced relaxation (Figure 5b); the highest concentration (10^{-5}M) produced a slight but statistically significant ($P < 0.05$) reduction in the carbachol response. In other experiments ($n = 3$), a still higher concentration of propranolol (10^{-4}M) entirely prevented carbachol-induced relaxation.

5-Hydroxytryptamine had purely relaxant actions in bullfrog hemilungs, and had no effect at all at concentrations below 10^{-5}M. A clear maximal effect was not elicited in most experiments, despite the high concentrations employed (Figure 6a). The highest concentration tested, 10^{-3}M, ($n = 9$; Figures 4 and 6) elicited relaxation equivalent to $24 \pm 4\%$ of I_{max}. Methysergide, 3×10^{-5}M, consistently reduced ($P < 0.01$) the response to 10^{-3}M 5-hydroxytryptamine to $45 \pm 12\%$ of that seen in matched hemilungs (Figure 6a), but did not signific-

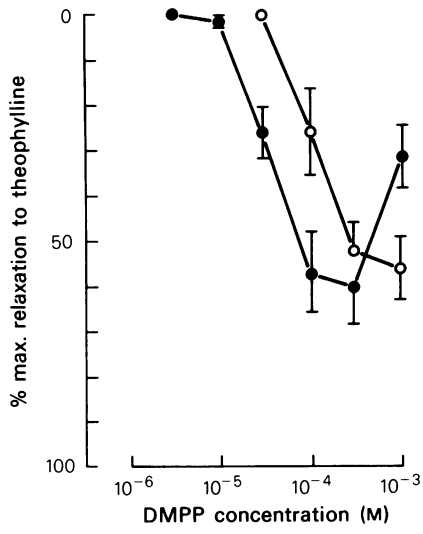

Figure 3 Cumulative concentration-effect curves for DMPP-induced (○) relaxation of bullfrog hemilungs. Hexamethonium, (10^{-3}M) (●) added 30 min before DMPP, surmountably antagonized DMPP-induced relaxation of matched hemilungs. Propranolol (10^{-6}M) and atropine (10^{-5}M) were added during the initial equilibration period. Means of., $n = 5$; vertical lines indicate s.e. DMPP: 1–1–dimethyl–4–phenylpiperazinium.

antly depress the response to carbachol (Figure 6b).

Hemilungs that were maximally relaxed with isoprenaline (10^{-4}M), or relaxed with 10^{-3}M adrenaline, noradrenaline or 5-hydroxytryptamine were further relaxed by addition of carbachol (Figure 7a and Table 3). The extent of further carbachol-induced relaxation was at least equal to that of the initially tested agonist.

Vasoactive intestinal peptide

From 0.1 to 3 μg/ml, vasoactive intestinal peptide ($n = 4$) produced a slight dose-related relaxation. At 3 μg/ml (Figure 4), relaxation corresponded to $8 \pm 2\%$ of I_{max}; this was not increased by raising the concentration to 6 μg/ml.

ATP and adenosine

At concentrations between 10^{-6} and 10^{-4}M, the only effect of ATP and adenosine was to elicit a slight contraction. Increasing the concentration to 10^{-3}M produced an initial contraction followed by some degree of relaxation in most preparations. The relaxant effect of 10^{-3}M ATP began at about 15 min after addition, and reached a peak effect at 30 to 120 min (Figure 8a). Peak relaxant effects ranged from 1 to 19% of I_{max} ($9 \pm 3\%$, $n = 5$), and subsequent addition of carbachol produced a further marked relaxation (Figure 8a, and Table 3). The relaxant effect of 10^{-3}M adenosine slowly increased throughout the experimental period, following an approximately exponential time course (Figure 9a). At 120 min after addition, adenosine-induced relaxation varied from 0 to 25% of I_{max} ($12 \pm 6\%$, $n = 5$). Addition of carbachol at this time (Figure 9a, and Table 3) produced a further, marked relaxation.

Pretreatment with 10^{-4}M indomethacin reduced or prevented the initial contractile response to ATP and adenosine, but caused a slow relaxation of the hemilungs (Figures 8b, and 9b). Hemilungs that received both indomethacin and either 10^{-3}M ATP (Figure 8b) or 10^{-3}M adenosine, (Figure 9b), showed a greater relaxation than that produced by ATP or adenosine alone. However, the time course for relaxation was much slower than the effect of carbachol (Figure 4). Addition of carbachol to these hemilungs (Figures 7–9) greatly accelerated the rate of relaxation.

Discussion

Three separate actions of carbachol

Carbachol produces its effects in bullfrog hemilungs through three separate actions. At low concentrations (10^{-8} to 3×10^{-6}M) stimulation of muscarinic receptors elicits contraction, which can be blocked by atropine. At intermediate concentrations (10^{-5} to 10^{-4}M) the predominant effect is relaxation, and at high concentrations ($> 10^{-4}$M) carbachol again elicits contraction. Neither of the last two responses are altered by pretreatment with high concentrations of atropine (Figure 2a).

Since the relaxant response to carbachol or DMPP is antagonized by hexamethonium, it represents an action at nicotinic receptors. The concentration of hexamethonium (10^{-3}M), which was required to shift the dose-response curve for carbachol-induced relaxation of hemilungs, is similar to the threshold concentration (3×10^{-3}M) for transmission block in bullfrog sympathetic ganglia (Kosay, Riker & Guerrero, 1972). Although some nicotinic receptors also might be present on smooth muscle or on secretory cells, such receptors play little or no role in the inhibitory response, since relaxation is almost completely prevented by tetrodotoxin (Figure 2c), which selectively blocks the fast sodium channel associated with nerve conduction (Narahashi, 1972). Tetrodotoxin has no effect on the responsiveness of smooth muscle (Kao, 1966; Bortoff & Muller, 1975), and only a slight effect on glandular secretion

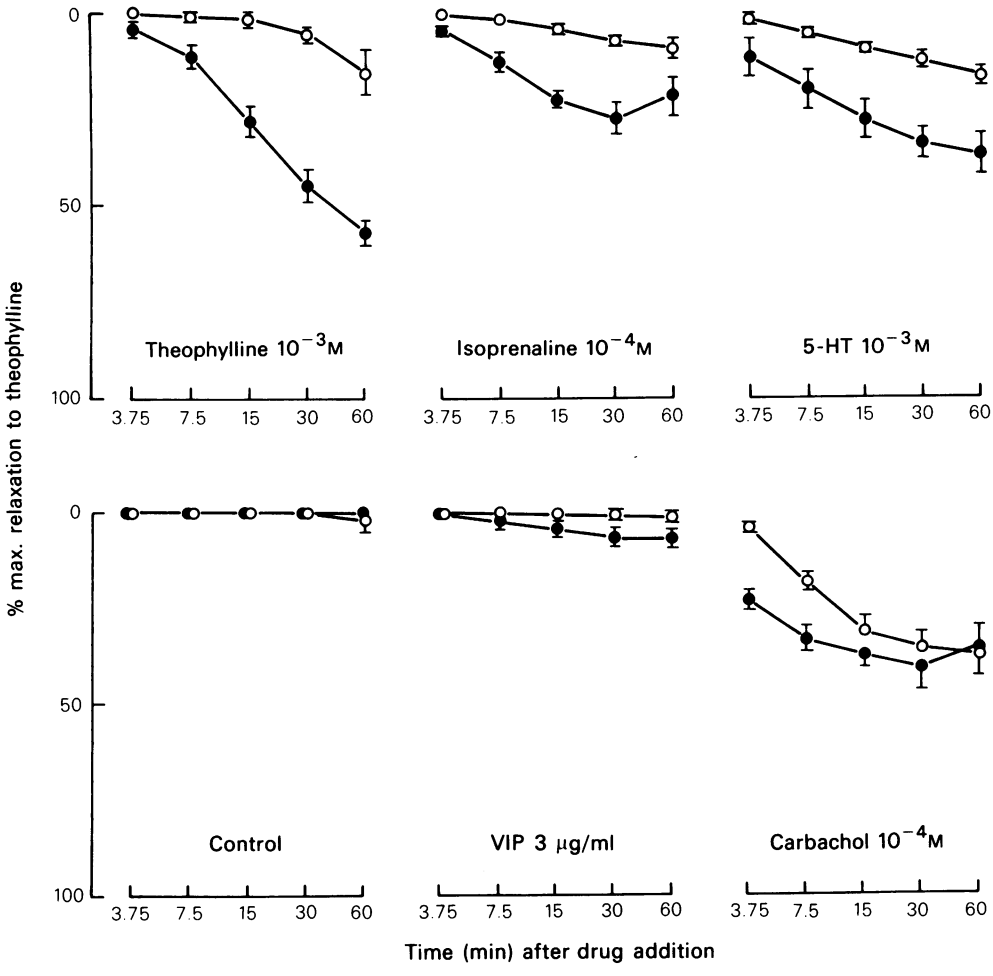

Figure 4 Time-action curves for agonist-induced relaxation of matched bullfrog hemilungs, in the presence (○) and absence (●) of 10^{-4}M procaine added 15 min before the agonist. Propranolol (10^{-6}M) and atropine (10^{-5}M) were added to the baths during the initial equilibration period; propranolol was omitted in isoprenaline experiments. Control hemilungs, with and without procaine, maintained a constant length throughout the experimental period. Each agonist was tested in a different series of matched hemilungs. Means of $n = 6$ for carbachol; $n = 4$ for theophylline, isoprenaline, 5-hydroxytryptamine (5-HT), control hemilungs and vasoactive intestinal peptide (VIP); vertical lines show s.e.

(Donatsch, Lowe, Richardson & Taylor, 1977; Kidokoro, Ritchie and Hagiwara, 1979).

The contractile response to high concentrations of carbachol ($> 10^{-4}$M) in the presence of atropine can be pharmacologically dissociated from the inhibitory response (Figures 1b and 2e) by 10^{-4}M procaine, which selectively blocks the contraction. Further, since a high dose contractile response is still evident in hemilungs pretreated with tetrodotoxin (Figure 2c), the contraction is not neurally mediated. APUD-like cells or monoamine-containing neuroepithelial bodies, which have been demonstrated in morphological studies of frog (Wasano & Yamamoto, 1978) and toad (Rogers & Haller, 1978) lungs, represent a possible site of action for this effect.

Distinctive characteristics of the relaxant response

Furchgott & Zawadzki (1980) have recently shown that acetylcholine-like agonists can relax vascular smooth muscle by releasing an unknown inhibitory mediator from endothelial cells of the intima. This effect is blocked by atropine. Carbachol-induced relaxation of bullfrog lung is fundamentally different in

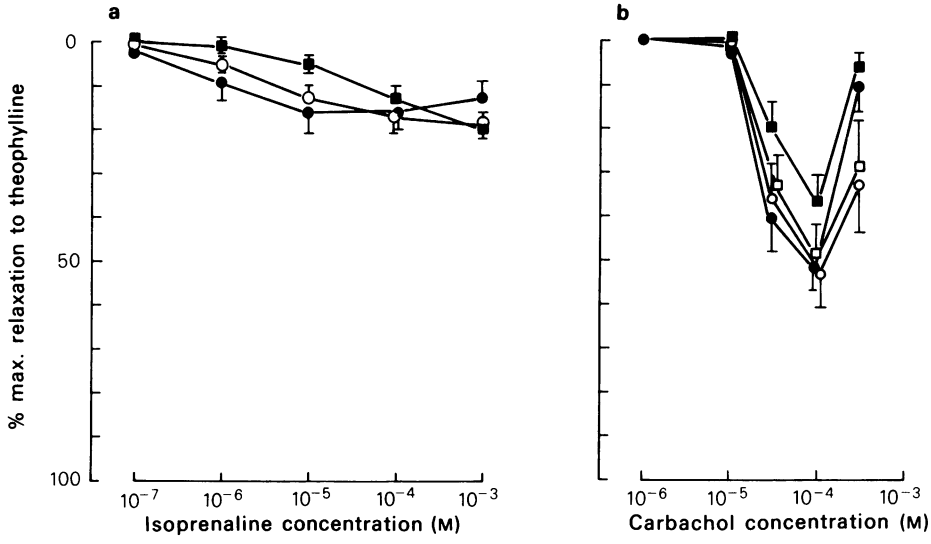

Figure 5 Effect of propranolol on isoprenaline- and carbachol-induced relaxation of bullfrog hemilungs. Isoprenaline effects were tested in one series of matched hemilungs (a), and carbachol in a separate series of matched hemilungs (b). Three concentrations of propranolol were tested in both series; the middle concentration (3×10^{-6}M), which produced an antagonism of isoprenaline of intermediate intensity, is not shown in (a). Propranolol concentrations: (●) none; (○) 10^{-6}M; (□) 3×10^{-6}M; (■) 10^{-5}M. Propanolol was added to the bath solution 60 min before testing the response to isoprenaline or carbachol. Atropine (10^{-5}M) was added during the initial equilibration period. Means of $n = 5$; vertical lines show s.e.

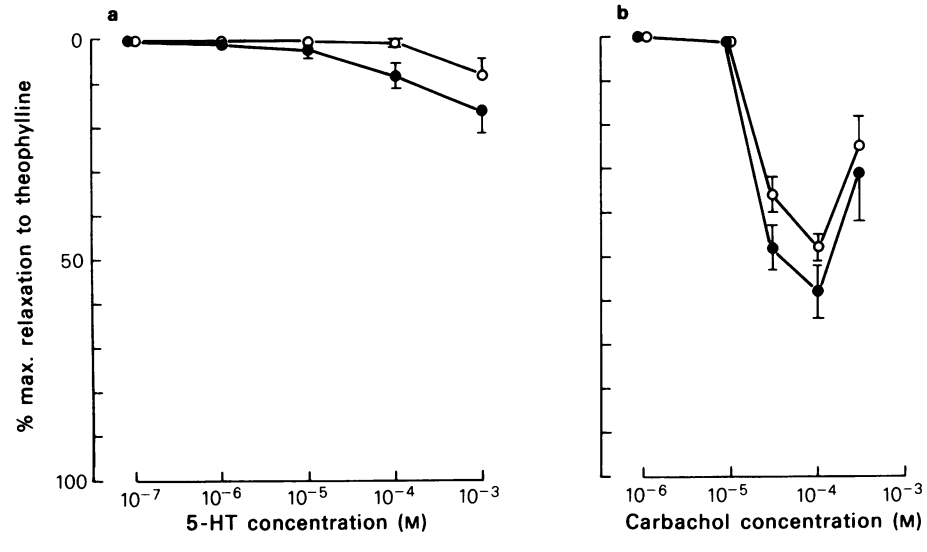

Figure 6 Effect of methysergide on 5-hydroxytryptamine (5-HT)- and carbachol-induced relaxation of bullfrog hemilungs. The effects of 5-HT (a) and carbachol (b) were tested in matched hemilungs from the same animals: (●) controls; (○) methysergide 3×10^{-5}M. Methysergide was added to the bath solution 30 min before testing the effects of 5-HT or carbachol. Atropine (10^{-5}M) was added during the initial equilibration period. Means of $n = 5$; vertical lines show s.e.

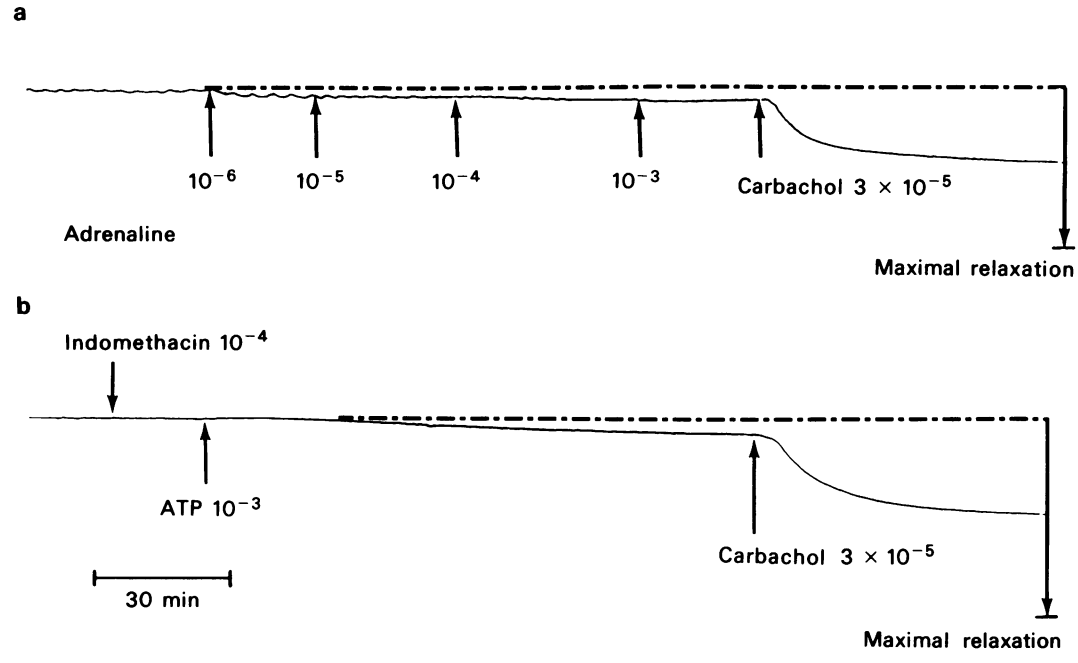

Figure 7 Carbachol-induced relaxation in bullfrog hemilungs partially relaxed with high concentrations of adrenaline (a) or indomethacin and ATP (b). Addition of drug is indicated by an arrow, with the cumulative drug concentration (M) appended. Large vertical arrows to the right show I_{max} as obtained after subsequent relaxation with theophylline (10^{-2}M). Propranolol (10^{-6}M) and atropine (10^{-5}M) were added during the initial equilibration period for (b) and atropine (10^{-5}M) alone for (a). In trace (a), cumulative addition of adrenaline from 10^{-6} to 10^{-3}M produced relaxation equivalent to 11% of I_{max}; subsequent addition of carbachol elicited a rapid relaxation of much greater intensity. In (b), the slow relaxation following addition of ATP achieved 10% of I_{max} after 120 min exposure; subsequent addition of carbachol elicited a rapid relaxation of much greater intensity. Addition of indomethacin alone in a matched hemilung (not shown) produced relaxation equivalent to 5% of I_{max} after 140 min exposure.

that it is elicited by stimulation of neural nicotinic receptors with release of an inhibitory neurotransmitter from postganglionic nerve terminals. The resulting inhibitory response has multiple distinguishing characteristics which serve as a fingerprint for the unknown inhibitory transmitter. These include a ceiling effect at 50% of I_{max}, a fast onset of action, and a distinctive pattern of drug interactions. Neither β-adrenoceptor agonists nor putative nonadrenergic inhibitory transmitters reproduce these characteristics.

Comparison with effects of other agonists

Bullfrog lung demonstrates a hierarchy of responses with marked differences in efficacy between inhibitory agonists. With the exception of theophylline, carbachol elicited the most intense relaxation of any

Table 3 Carbachol-induced relaxation* in preparations already relaxed with other agonists

	Agonist alone	*Agonist + carbachol* 3×10^{-5}M	*Agonist* + carbachol 10^{-4}M
Adrenaline 10^{-3}M†	14 ± 2	66 ± 2	68 ± 2
Noradrenaline 10^{-3}M†	19 ± 2	68 ± 3	69 ± 3
Isoprenaline 10^{-4}M†	23 ± 5	50 ± 8	59 ± 7
5-Hydroxytryptamine 10^{-3}M‡	32 ± 4	47 ± 3	64 ± 3
ATP 10^{-3}M‡	9 ± 3¶	40 ± 6	-
Adenosine 10^{-3}M‡	12 ± 6¶	59 ± 8	-

* % of I_{max}, mean ± s.e. (n = 5 to 7)
†pretreated with atropine 10^{-5}M; ‡pretreated with atropine 10^{-5}M and propranolol 10^{-6}M.
¶Two hours incubation, time courses shown in Figures 8 and 9.

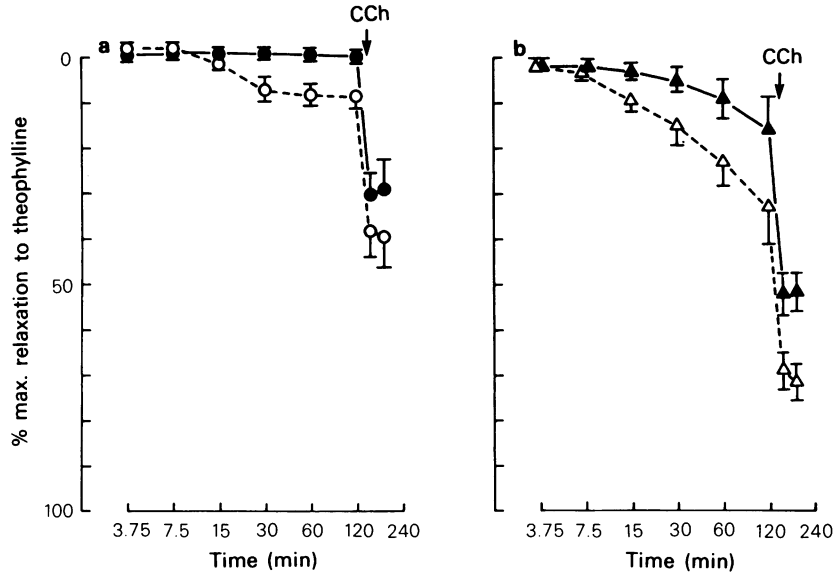

Figure 8 ATP-induced relaxation, with (b) and without (a) indomethacin pretreatment. Propranolol (10^{-6}M) and atropine (10^{-5}M) were added during the initial equilibration period and carbachol (CCh, 3×10^{-5}M) at the end of the experiment. Matched hemilungs treated with no other drug (a,●), ATP 10^{-3}M (a,○), indomethacin 10^{-4}M (b,▲), or indomethacin 10^{-4}M plus ATP 10^{-3}M (b,△). Horizontal axes show time after addition of ATP or equivalent elapsed time in the parallel control series (control or indomethacin alone). In series (a), ATP elicited a biphasic response with delayed relaxation; subsequent addition of carbachol elicited a rapid relaxation of much greater intensity. In series (b), indomethacin was added 20 min before the time of addition of ATP; indomethacin by itself produced a slow relaxation; subsequent addition of ATP produced relaxation with little or no initial contraction; and carbachol accelerated the rate of relaxation. Means with s.e., $n = 5$.

agonist tested. In comparison, catecholamines (Figures 5 and 7a) 5-hydroxytryptamine (Figure 6), ATP (Figure 8) and adenosine (Figure 9) required very high concentrations (10^{-3}M) to elicit effects that were consistently less than those of carbachol. Responses to vasoactive intestinal peptide were barely detectable. Thus, simply on the basis of intensity of the response, it is unlikely that any of the agonists tested plays a major role in carbachol-induced relaxation. This conclusion is further supported by the ability of carbachol to produce a marked, further relaxation in preparations that are already relaxed with high concentrations of catecholamines, 5-hydroxytryptamine, ATP or adenosine (Figures 7–9, Table 3).

In addition to its greater intensity, carbachol-induced relaxation occurred more rapidly than that of any other agonist (Figures 4, 8 and 9). In part, this may be explained by release of the endogenous inhibitory transmitter in close proximity to its site of action. However, the much slower time course for relaxation following addition of ATP (Figure 8) or adenosine (Figure 9) are unlikely to result from access limitations alone, and probably reflect actions mediated by fundamentally different effector pathways.

ATP and adenosine usually produced an initial contraction preceding relaxation. Adenine nucleotides are known to increase synthesis of prostaglandins (Needleman, Minkes, & Douglas, 1974) and high concentrations of indomethacin (2 to 5×10^{-5}M) have been shown to block the 'rebound contraction' following stimulation of non-adrenergic, non-cholinergic inhibitory nerves (Burnstock, Cocks, Paddle & Staszewska-Barczak, 1975). Since increased formation of stimulant prostaglandins could obscure the intrinsic relaxant activity of purine derivatives, hemilungs were pretreated with 10^{-4}M indomethacin to inhibit prostaglandin synthesis (Ferreira, Moncada & Vane, 1971). This reduced the contractile responses to ATP and adenosine, but did not enhance their relaxant effects, which were approximately additive with those of indomethacin.

Drug interactions

The interpretation of propranolol effects in bullfrog hemilungs is complicated on the one hand by the very high concentrations of catecholamines needed to elicit relaxation, and on the other by the nonspecific depressant or quinidine-like effects of high concentrations of propranolol. Low concentrations (10^{-6}M

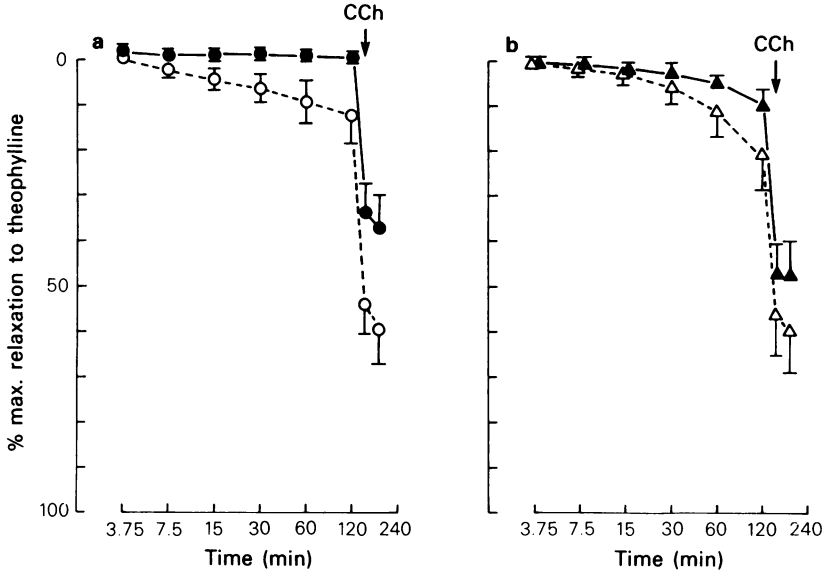

Figure 9 Adenosine-induced relaxation, with (b) and without (a) indomethacin pretreatment. Propranolol (10^{-6}M) and atropine (10^{-5}M) were added during the initial equilibration period and carbachol (CCh, 3×10^{-5}M) at the end of the experiment. Matched hemilungs treated with no other drug (a,●), adenosine 10^{-3}M (a,○), indomethacin 10^{-4}M (b,▲), or indomethacin 10^{-4}M plus adenosine 10^{-3}M (b,△). Horizontal axis show time after addition of adenosine or equivalent elapsed time in parallel control series (control or indomethacin alone). In series (a), adenosine elicited a slow relaxation following an exponential time course; subsequent addition of carbachol elicited a rapid relaxation of much greater intensity. In series (b), indomethacin was added 20 min before addition of adenosine; indomethacin by itself produced a slow relaxation; indomethacin plus adenosine produced approximately additive effects; subsequent addition of carbachol accelerated the rate of relaxation. Means with s.e., $n = 5$.

and 3×10^{-6}M) of propranolol antagonized the relaxant effects of a low concentration of isoprenaline (10^{-6}M), but did not impair carbachol-induced relaxation (Figure 5). Similar low concentrations of propranolol have been shown to antagonize the β-adrenergic effects of nerve stimulation in the isolated innervated frog heart preparation (Tripathi & Gambhir, 1978), and to block exercise-induced tachycardia in man (Chidsey, Pine, Favrot, Smith, Leonetti, Morselli & Zanchetti, 1976).The lack of effect of low concentrations of propranolol on carbachol-induced relaxation is further evidence that release of endogenous catecholamines makes little or no contribution to the carbachol response. As the propranolol concentration was increased to 10^{-5}M and 10^{-4}M, carbachol-induced relaxation was slightly reduced and blocked, respectively. Since this is the concentration range in which, nonspecific depressant effects of propranolol have been demonstrated in heart (Morales-Aguilerá & Vaughan Williams, 1965; Davis & Temte, 1968; Coltart & Meldrum, 1971; Tarr, Luckstead, Jurewicz & Haas, 1973) and nerve (Wu & Narahashi 1973), the inhibition of carbachol-induced relaxation by high concentrations of propranolol probably represents an action unrelated to β-adrenoceptor blockade.

With the exception of quinidine, drugs affecting autacoid pathways had no effect on carbachol-induced relaxation. Carbachol responses in the presence of indomethacin were at least as great as in control hemilungs (Figures 8 and 9), indicating that prostaglandin formation did not contribute to the inhibitory effect. Dipyridamole (Figure 2f), which inhibits adenosine uptake (Kolassa, Pfleger & Rummel, 1970) and enhances the relaxant effects of adenosine, ATP and the non-adrenergic inhibitory system in guinea-pig trachealis muscle (Coleman & Levy, 1974; Coleman, 1976), did not alter the carbachol response in bullfrog lung. Methysergide (Figure 6) also was ineffective against carbachol-induced relaxation.

Carbachol-induced relaxation can be blocked either by drugs such as hexamethonium and tetrodotoxin that act on the neural pathways leading to release of the inhibitory transmitter, or by drugs that act directly on smooth muscle to depress its response to the transmitter. Quinidine has been shown to antagonize 'purinergic inhibition' in some preparations (Burnstock, 1972), but the quinidine block of carbachol-induced relaxation (Figure 2d) could represent a depressant action at ganglia or nerve terminals rather than antagonism of the inhibitory trans-

mitter. The concentration of quinidine, 3×10^{-4}M, that was needed to prevent carbachol-induced relaxation has been shown to suppress sodium and potassium conductance changes in squid giant axon (Yeh & Narahashi, 1976).

At local anaesthetic concentrations, procaine also blocks carbachol-induced relaxation. However, at a lower concentration, 10^{-4}M, procaine spares the relaxant response to carbachol while greatly retarding responses to other inhibitory agonists. This procaine resistance may be particularly useful as a marker of the inhibitory transmitter in bullfrog lung, since the differential effect clearly is at muscle rather than at nerve. It should be emphasized that the high potency stimulant effect of procaine in bullfrog lung is an anomalous response of unknown mechanism, which is not typical of effects in mammalian tissues, and which is dependent on a procaine-like structure rather than local anaesthetic activity (Downes & Taylor, 1982). Since the procaine contracted lung shows retarded responses to a wide variety of inhibitory agonists (Figure 4), the antagonism is independent of specific receptors and probably involves an intracellular effector pathway common to the action of many inhibitory agonists. This pathway is not essential to the action of the inhibitory transmitter released by nicotinic stimulation, which elicits a rapid relaxation in both control and procaine-treated preparations.

In summary, nicotinic agonists elicit a neurally mediated relaxation of bullfrog hemilungs that greatly exceeds the relaxant effects of exogenously administered catecholamines or the putative transmitters for the nonadrenergic inhibitory system. The relaxant effect appears to involve release of an inhibitory transmitter from postganglionic neurones. This transmitter, which remains to be identified, operates independently of adrenergic, 5-hydroxytryptaminergic or purinergic pathways.

This study was supported in part by NIH Grant ROI GM 24524, and by a grant from the Medical Research Foundation of Oregon.

References

BORTOFF, A. & MULLER, R. (1975). Stimulation of intestinal smooth muscle by atropine, procaine, and tetrodotoxin. *Am. J. Physiol.*, **229,** 1609–1613.

BRECHT, K. & FRAESSLE, K. (1944). Über die wirkung elektrischer reizung des vagosympathicus auf die froschlunge. *Pflügers Arch.*, **247,** 649–659.

BURNSTOCK, G. (1969). Evolution of the autonomic innervation of visceral and cardiovascular systems in vertebrates. *Pharmac. Rev.*, **21,** 247–324.

BURNSTOCK, G. (1972). Purinergic nerves. *Pharmac. Rev.*, **24,** 509–581.

BURNSTOCK, G., COCKS, T., PADDLE, B. & STASZEWSKA-BARCZAK, J. (1975). Evidence that prostaglandin is responsible for the 'rebound contraction' following stimulation of non-adrenergic, non-cholinergic ('purinergic') inhibitory nerves. *Eur. J. Pharmac.*, **31,** 360–362.

CAMPBELL, G. (1971). Autonomic innervation of the lung musculature of a toad (*Bufo marinus*). *Comp. gen. Pharmac.*, **2,** 280–286.

CAMPBELL, G. & DUXSON, M.J. (1978). The sympathetic innervation of lung muscle in the toad *Bufo marinus*: A revision and an explanation. *Comp. Biochem. Physiol.*, **60C,** 65–73.

CAMPBELL, G., HALLER, C.J. & ROGERS, D.C. (1978). Fine structural and cytochemical study of the innervation of smooth muscle in an amphibian (*Bufo marinus*) lung before and after denervation. *Cell Tiss. Res.*, **194,** 419–432.

CARLSON, A.J. & LUCKHARDT, A.B. (1920). Studies on the visceral sensory nervous system – 1. Lung automatism and lung reflexes in the frog. (*R. pipiens and R. catesbiana*). *Am. J. Physiol.*, **54,** 55–95

CHIDSEY, C., PINE, M., FAVROT, L., SMITH, S., LEONETTI, G. MORSELLI, P. & ZANCHETTI, A. (1976). The use of drug concentration measurements in studies of the therapeutic response to propranolol. *Postgrad. med. J.*, **52** (Suppl. 4), 26–32.

COLEMAN, R.A. & LEVY, G.P. (1974). A non-adrenergic inhibitory nervous pathway in guinea-pig trachea. *Br. J. Pharmac.*, **52,** 167–174.

COLEMAN, R.A. (1976). Effects of some purine derivatives on the guinea-pig trachea and their interaction with drugs that block adenosine uptake. *Br. J. Pharmac.*, **57,** 51–57.

COLTART, D.J. & MELDRUM S.J. (1971) The effect of racemic propranolol, dextro-propranolol and racemic practolol on the human and canine cardiac transmembrane action potential (beta blockade and the cardiac action potential). *Archs Int. Pharmacodyn.*, **192,** 188–197.

CONWAY, E.J. (1957). Nature and significance of concentration relations of potassium and sodium ions in skeletal muscle. *Physiol. Rev.*, **37,** 84–132.

DAVIS, L.D. & TEMTE, J.V. (1968). Effects of propranolol on the transmembrane potentials of ventricular muscle and purkinje fibers of the dog. *Circulation Res.*, **22,** 661–677.

DOHADWALLA, A.N., FREEDBERG, A.S. & VAUGHAN WILLIAMS, E.M. (1969). The relevance of β-receptor blockade to ouabain-induced cardiac arrhythmias. *Br. J. Pharmac.*, **36,** 257–267.

DONATSCH, P., LOWE, D.A., RICHARDSON, B.P. & TAYLOR, P. (1977). The functional significance of sodium channels in pancreatic beta-cell membranes. J. Physiol., **267,** 357–376.

DOWNES, H. & TAYLOR, S.M. (1982). A potent, structurally specific stimulant action of procaine in bullfrog lung. *Comp. Biochem. Physiol.*, **72C,** No. 1, 39–43.

FENN, W.O. (1936). Electrolytes in muscle. *Physiol. Rev.*, **16,** 450–487.

FERREIRA, S.H., MONCADA, S. & VANE, J.R. (1971). Indomethacin and aspirin abolish prostaglandin release from the spleen. *Nature New Biol.*, **231,** 237–239.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288,** 373–376.

HOLMGREN, S. & CAMPBELL, G. (1978). Adrenoceptors in the lung of the toad *Bufo marinus*: Regional differences in responses to amines and to sympathetic nerve stimulation. *Comp. Biochem. Physiol.*, **60C,** 11–18.

HOWELL, B.J., BAUMGARDNER, F.W., BONDI, K. & RAHN, H. (1970). Acid-base balance in cold-blooded vertebrates as a function of body temperature. *Am. J. Physiol.*, **218,** 600–606.

KAO, C.Y. (1966). Tetrodotoxin, saxitoxin and their significance in the study of excitation phenomena. *Pharmac. Rev.*, **18,** 997–1049, 1966.

KIDOKORO, Y., RITCHIE, A.K. & HAGIWARA, S. (1979). Effect of tetrodotoxin on adrenaline secretion in the perfused rat adrenal medulla. *Nature*, **278,** 63–65.

KOBAYASI, S. & YODA, C. (1960). Effects of adrenaline and acetylcholine upon the smooth muscle of isolated lungs of Japanese toads. *Acta med. biol., Niigata*, **8,** 241–250.

KOLASSA, N., PFLEGER, K. & RUMMEL, W. (1970). Specificity of adenosine uptake into the heart and inhibition by dipyridamole. *Eur. J. Pharmac.*, **9,** 265–268.

KOSAY, S., RIKER, W.K. & GUERRERO, S. (1972). Effects of d-tubocurarine on the frog sympathetic ganglion cell and on synaptic function. *J. Pharmac. exp. Ther.*, **180,** 255–264.

MATSUZAKI, Y., HAMASAKI, Y. & SAID, S.I. (1980). Vasoactive intestinal peptide: A possible transmitter of nonadrenergic relaxation of guinea-pig airways. *Science*, **210,** 1252–1253.

MEVES, H. (1953). Die wirkung von adrenalin und adrenalinverwandten auf gefäbe und muskulatur der froschlunge. *Pflügers Arch.*, **258,** 200–210.

MORALES-AGUILERÁ, A. & VAUGHAN WILLIAMS, E.M. (1965). The effects on cardiac muscle of β-receptor antagonists in relation to their activity as local anesthetics. *Br. J. Pharmac.*, **24,** 332–338.

NARAHASHI, T. (1972). Mechanism of action of tetrodotoxin and saxitoxin on excitable membranes. *Fedn Proc*, **31,** 1124–1132.

NEEDLEMAN, P., MINKES, M.S. & DOUGLAS, J.R. (1974). Stimulation of prostaglandin biosynthesis by adenine nucleotides. *Circulation Res.*, **34,** 455–460.

ROBINSON, P.M., McLEAN, J.R. & BURNSTOCK, G. (1971). Ultrastructural identification of non-adrenergic inhibitory nerve fibers. *J. Pharmac. exp. Ther.*, **179,** 149–160.

ROGERS, D.C. & HALLER, C.J. (1978). Innervation and cytochemistry of the neuroepithelial bodies in the ciliated epithelium of the toad lung (*Bufo marinus*). *Cell Tiss. Res.*, **195,** 395–410

SCHNIZER, W., HOANG, N.-D., & BRECHT, K. (1968). Transmitter in der froschlunge. *Pflügers Arch.*, **304,** 271–283.

TARR, M., LUCKSTEAD, E.F., JUREWICZ, P.A. & HAAS, H.G. (1973). Effect of propranolol on the fast inward sodium current in frog atrial muscle. *J. Pharmac. exp. Ther.*, **184,** 599–610.

TRIPATHI, H.C. & GAMBHIR, S.S. (1978). Innervated Straub's heart – A simple method for adrenergic neurone blocking studies. *Indian J. med. Res.*, **67,** 1051–1054.

WASANO, K. & YAMAMOTO, T. (1978). Monoamine-containing granulated cells in the frog lung. *Cell Tiss. Res.*, **193,** 201–209.

WOOD, M.J. & BURNSTOCK, G. (1967). Innervation of the lungs of the toad (*Bufo marinus*) -- 1. Physiology and Pharmacology. *Comp. Biochem. Physiol.*, **22,** 755–766.

WU, C.H. & NARAHASHI, T. (1973). Mechanism of action of propranolol on squid axon membranes. *J. Pharmac. exp. Ther.*, **184,** 155–162.

YEH, J.Z. & NARAHASHI, T. (1976). Mechanism of action of quinidine on squid axon membranes. *J. Pharmac. exp. Ther.*, **196,** 62–70.

(*Received July 29, 1982.*)