Br. J. Pharmac. (1984), **81**, 449–455

Influence of plasma protein on the inhibitory effects of indocyanine green and bromcresol green on pulmonary prostaglandin E_1 extraction

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1 The purpose of this study was to examine the influence of plasma protein on the inhibitory effects of the anionic dyes indocyanine green and bromcresol green on prostaglandin E_1 (PGE_1) uptake by the lungs.

2 Dog lung lobes were isolated and perfused with either autologous plasma or Krebs-Ringer bicarbonate solution (KRB) containing no protein but with dextran used as a colloid.

3 PGE_1 uptake was determined by injecting a bolus, containing radiolabelled PGE_1 into the lobar artery and then analysing ethanolic extracts of the venous effluent for radioactivity in PGE_1 and PGE_1 metabolites by thin layer chromatography and scintillation counting.

4 When the lobes were perfused with KRB, bromcresol green at an average initial concentration of 28.5 μM, reduced PGE_1 by an average of 56%. When the lobes were perfused with plasma, similar concentrations of bromcresol green reduced the uptake by less than 2%.

5 A similar result was obtained with indocyanine green, which at an average initial concentration of 17.5 μM reduced uptake by about 70% when the lobes were perfused with KRB, but when the lobes were perfused with plasma similar concentrations of the dye reduced uptake by less than 3.5%.

6 The results suggest that plasma protein binding interferes with the inhibitory effects of these dyes on PGE_1 uptake in the lungs.

Introduction

Certain organic anionic dyes have been found to inhibit prostaglandin E_2 and $F_{2\alpha}$ uptake by isolated lungs perfused with physiological salt solutions (Bito & Baroody, 1975; Bito *et al.*, 1977; Bakhle *et al.*, 1978; Bakhle, 1981). Some of these dyes; for example, indocyanine green and the sulphonphthaleins, of which bromcresol green is an example, bind to plasma proteins (Waldmann-Meyer & Schilling, 1956; Fox *et al.*, 1957; Cherrick *et al.*, 1960; Fox & Wood, 1960; Baker, 1966; Janecki & Krawcynski, 1970; Kawasaki *et al.*, 1973; Deutschmann *et al.*, 1974; Kamisaka *et al.*, 1974)

In fact, in the case of indocyanine green, its high affinity for plasma proteins is one of the characteristics which makes it clinically useful as an intravascular indicator for indicator dilution studies (Fox *et al.*, 1957; Fox & Wood, 1960). Hellewell & Pearson (1982) found that sulphasalazine, which also binds to plasma proteins, was an inhibitor of $PGF_{2\alpha}$ uptake by neonatal pig lungs perfused with protein-free perfusate, but was ineffective when the perfusate contained albumin. Therefore, in the present study, we examined how the presence or absence of plasma protein influences the inhibitory effects of indocyanine green and bromcresol green dyes on the extraction of PGE_1 by the dog lung.

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Methods

The experiments were carried out on dog isolated perfused lung lobes which could be perfused with

either autologous plasma or a modified Krebs-Ringer bicarbonate solution. The details of the lung lobe perfusion procedure have been described previously (Rickaby *et al.*, 1981). In brief, each dog (mean weight $\pm$s.d. = 21.9 $\pm$ 5.5 kg, n = 16 dogs) was anaesthetized with pentobarbitone sodium (30 mg kg^{-1}i.v.), heparinized (1,250 iu kg^{-1}) and exsanguinated via a carotid artery. Approximately 1.3 l of the autologous blood were centrifuged (1,000 $\times$ g) and the plasma fraction used to prime the perfusion system. The left lower lobe artery, vein and bronchus were cannulated and the lobe was removed and placed in a perfusion chamber. The vascular cannulae were attached to the perfusion system and the bronchial cannula to the ventilation system.

The perfusion system was a recirculating system maintained at 37°C. The plasma was pumped (Masterflex roller pump) into the lobar artery from a reservoir into which the plasma drained from the lobar vein. The perfusion was begun at a slow flow rate and the first 400 ml of the effluent plasma mixed with residual blood from the lobe were collected and recentrifuged. The plasma flow rate was finally raised to a level which remained constant in a given experiment, and which averaged 308 $\pm$ 30 ml min^{-1} (mean $\pm$ s.d.) for all experiments. Once the perfusion rate had been set, we proceeded to study the plasma perfused lobe, or we changed the lobe perfusate to a Krebs-Ringer bicarbonate solution (KRB) containing 5% dextran (average mol. wt. 80,000) and 5.5 mM glucose (Linehan *et al.*, 1981). The first 500 ml of effluent KRB were collected from the venous cannula and discarded to remove as much residual plasma as was practical. Other than the perfusate composition, the lobes were treated the same. The total recirculating perfusate (plasma or KRB) volume ranged from 475 ml to 775 ml. The lobes were ventilated by a Harvard piston respirator with a gas mixture containing approximately 16% O_2 and 5.8% CO_2 in N_2. The tidal volume was 150 ml. The frequency was 8 breaths/min, and end-inspiratory and end-expiratory pressures were about 5 and 1.5 Torr, respectively. This provided plasma P_{O2}, P_{CO2} and pH of 106 $\pm$ 5 Torr, 39.1 $\pm$ 1.6 Torr, and 7.39 $\pm$ 0.03, respectively, or KRB P_{O2}, P_{CO2} and pH of 107 $\pm$ 5 Torr, 36.5 $\pm$ 2.9 Torr and 7.37 $\pm$ 0.05, respectively (values are expressed as mean $\pm$ s.d). The arterial perfusion pressure was 5.3 $\pm$ 1.6 Torr during plasma perfusion and 5.4 $\pm$ 1.0 Torr during KRB perfusion with the venous pressure set at approximately 1 Torr. The pressures were measured relative to the top of the lobe at end expiration. None of the lung lobes appeared to be oedematous on gross examination at the end of the perfusion period. The wet weights of the lobes which were perfused only with plasma averaged 1.77 $\pm$ 0.10 g kg^{-1} dog body weight (mean $\pm$ s.e.mean), while those lobes which were also perfused with KRB averaged 1.93 $\pm$ 0.10 g kg^{-1}.

An injector was situated in the tubing leading to the arterial cannula so that a bolus (0.4 to 1.0 ml) containing 0.3 μCi [^{14}C]-prostaglandin E_1 (PGE_1) or 2–6 μCi [^{3}H]-PGE_1 and varying amounts of unlabelled PGE_1 could be rapidly injected. In most of the experiments, the bolus contained less than 8 nmol of PGE_1 which we considered to be a trace dose. However, in some experiments unlabelled PGE_1 was added to provide a dose of from 94 to 565 nmol. In preparing the bolus, the ethanolic stock solution containing the appropriate quantity of PGE_1 was dried under N_2 before being redissolved in the aqueous solution which was to be injected. The venous outflow was connected so that it could be diverted into the sampling tubes of a Gilson Escargot fraction collector just before the bolus injection. In a typical experiment, the ventilator was turned off at end expiration, and the sample collector was started at a rate of either 1.0 or 1.67 samples/s. The bolus was injected and sequential samples were collected for about 25 s. Internal standards were then prepared by adding a small quantity (10–50 μl) of the solution to be injected to samples collected before the appearance of the bolus in the venous effluent. Twelve ml of ethanol at 0°C were added to each sample, including the standards, in preparation for analysis as previously described (Dawson *et al.*, 1975; Cozzini & Dawson, 1977a). One ml of supernatant was analysed for ^{3}H or ^{14}C by liquid scintillation counting. Ten ml were dried under nitrogen, redissolved in 0.5 ml chloroform : methanol (4:1, v/v) solution and streaked on silica gel thin layer chromatography plates for separation of the PGE_1 from its metabolites formed in the lung lobe. The plates were developed for 15 cm in chloroform : methanol : acetic acid, 90:5:5 (v/v) and the radioactivity containing zones quantified using a Packard radiochromatogram scanner (Model 7201) with disc integrator. The ratio of PGE_1 to its metabolites and the total radioactivity in each sample were used to determine the fraction of the injected PGE_1 in each sample which reached the venous effluent without being taken up and/or metabolized in the lobe. The fraction of the injected PGE_1 extracted by the lung lobe (the PGE_1 extraction) was the difference between the amount of PGE_1 injected and the total unmetabolized PGE_1 recovered in all of the samples.

In the basic experimental protocol, an injection of PGE_1 was made and then the indocyanine green or bromcresol green dye, dissolved in 1–2 ml of distilled water, was added to the perfusion system and allowed to recirculate for 3–5 min. The same dose of PGE_1 was then injected again. In ten lobes, the injections were carried out while the lobe was perfused with either plasma or KRB. In four lobes, the injections

Table 1 PGE_1 extraction by plasma and KRB perfused lung lobes with and without bromcresol green dye

	Plasma perfusion PGE_1 extraction					*KRB perfusion PGE_1 extraction*			
Lobe no.	*Without dye*	*With dye*	*% decrease*	*Dye conc.(μM)*	*Lobe no.*	*Without dye*	*With dye*	*% decrease*	*Dye conc.(μM)*
2	90.2%	89.1%	1.2%	43.0	2	90.2%	33.7%	62.6%	43.0
5	90.7	86.6	4.5	45.2	3	88.5	34.9	60.6	36.8
11[1]	83.8	83.1	0.8	20.5	9	89.1	76.1	14.6	12.5
14	91.4	90.9	0.5	37.0	10	90.5	27.8	69.3	29.6
15	90.4	88.8	1.8	33.4	11[1]	91.2	25.8	71.7	20.5
Mean	89.3	87.7	1.8	35.8	Mean	89.9	39.7	55.8	28.5

[1] 94.6 nmol dose. In all other cases, trace doses were used.

were carried out while the lobe was perfused with plasma and then again after the perfusate was changed to KRB. In two of the lobes, the indocyanine green (645 nmol) was included in the second bolus rather than being equilibrated in the perfusion system. In these experiments, the boluses also included a tracer quantity of [^{14}C]-dextran (0.4 μCi and 0.33 μg with mol. wt. 60,000 to 90,000) as an intravascular indicator for comparison with the indocyanine green dye, and an aliquot of plasma or KRB was removed from each sample for liquid scintillation counting before the ethanol was added. In one lobe, no dye was added, instead, three PGE_1 injections of varying doses were made during plasma perfusion and then again during KRB perfusion.

Results

Tables 1 and 2 show the influence of bromcresol green and indocyanine green, respectively, on the amount of PGE_1 extracted from the perfusate as the PGE_1 passed through the lung lobe. In the concentrations used, both dyes had a marked inhibitory effect on the PGE_1 extraction by the KRB perfused lobes. On the other hand, in the plasma perfused lungs, the PGE_1 extraction was only slightly reduced when the dye was present. It is noted that this reduction in extraction was quite small in comparison to that in the KRB perfused lungs. The difference between the KRB and plasma perfused lungs is further demonstrated by Figures 1 and 2, in which the concentra-

Table 2 PGE_1 extraction by the plasma and KRB perfused lung lobes with and without indocyanine green dye

	Plasma perfusion PGE_1 extraction					*KRB perfusion PGE_1 extraction*			
Lobe no.	*Without dye*	*With dye*	*% decrease*	*Dye conc.(μM)*	*Lobe no.*	*Without dye*	*With dye*	*% decrease*	*Dye conc.(μM)*
1	93.4%	92.6%	0.8%	26.6	4	91.2%	20.6%	77.4%	17.0
6	92.2	86.3	6.4	26.6	7	93.1	14.8	84.1	17.8
7	90.8	89.5	1.4	25.8	8	90.6	24.2	73.3	28.9
12[1,2]	68.9	65.0	5.7	29.6	13[2]	96.9	57.9	40.2	5.7 (27.3)
12[2]	87.4	85.8	1.8	31.0	15	89.7	20.6	77.0	18.3
Mean	86.5	83.8	3.1	27.9	Mean	92.3	27.6	70.4	17.5

[1] 390 nmol dose. In all other cases, trace doses were used.
[2] The indocyanine green dye (645 nmol) was included in the bolus, in which case the dye concentration refers to the peak concentration in the venous effluent. In the KRB perfused lobe, no. 13, the value in parentheses is the peak venous dye concentration had there been no dye uptake by the lobe, calculated from the venous [^{14}C]-dextran concentration, Figure 3.

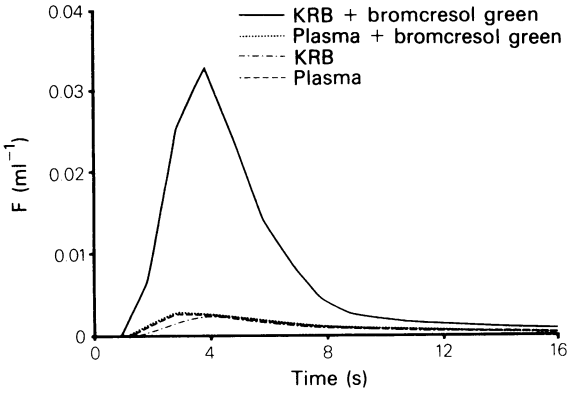

Figure 1 The effect of bromcresol green on the fraction (F) of the injected PGE_1 found in each ml of venous effluent following injection of PGE_1 into the artery of one lung lobe during perfusion with plasma or Krebs-Ringer bicarbonate solution (KRB). The fraction of the injected PGE_1 in the venous effluent was clearly increased by bromcresol green only when the lobe was perfused with KRB. Time in this figure and in Figures 2 and 3 is the time following the injection minus the transit time through the perfusion system from injection site to sampling site.

tions of PGE_1 in the venous effluent are shown as a function of time. The normalized concentration, F, is obtained by dividing the concentration in each sample by the amount of PGE_1 injected. The outflow concentrations of PGE_1 for the lungs perfused with KRB, plasma, or plasma plus dye were very small, while the reduction in the PGE_1 extraction when dye

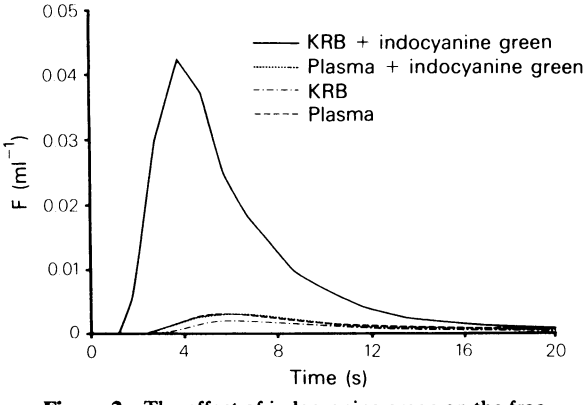

Figure 2 The effect of indocyanine green on the fraction (F) of the injected PGE_1 found in each ml of venous effluent following injection of PGE_1 into the artery of one lung lobe during perfusion with plasma or KRB. The fraction of the injected PGE_1 in the venous effluent was clearly increased by indocyanine green only when the lobe was perfused with KRB.

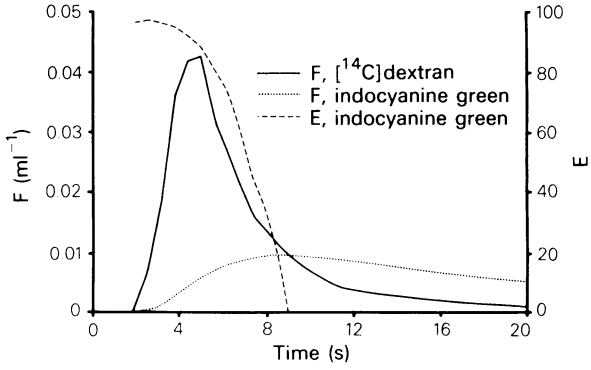

Figure 3 The fraction (F) of [^{14}C]-dextran and indocyanine green dye in each ml of venous effluent following their injection into the artery of one lung lobe perfused with KRB. The instantaneous extraction ratio (E) is also shown and demonstrates that the indocyanine green permeated the lobe rapidly and reversibly.

was added to the KRB perfusate resulted in a peak concentration of PGE_1 which was at least an order of magnitude greater than in the other three conditions.

In three of the experiments cited in Table 2, the indocyanine green was included within the injected bolus rather than being pre-equilibrated in the perfusion system. Again, the inhibitory effect was greatest in KRB perfused lobes. In some of the experiments, we used larger doses of PGE_1. This resulted in lower extractions in the plasma perfused lobes, but the difference in the inhibitory effects of the indocyanine green or bromcresol green between plasma and KRB was still evident.

One difference between plasma and KRB perfused lobes which we observed was that the KRB perfused lobes became noticeably coloured by the dyes while the plasma perfused lobes did not. Because indocyanine green is a clinically important substance, we decided to examine further its uptake by the lung lobes. This was accomplished by those experiments in which indocyanine green dye was included in the bolus. In addition to the PGE_1 and dye, [^{14}C]-dextran was included as an intravascular tracer to allow for the calculation of the instantaneous extraction ratio (E) of the dye, where $E = [1-(F_{dye}/F_{dex})] \times 100$; where F_{dex} is the normalized concentration of [^{14}C]-dextran and F_{dye} is the normalized concentration of indocyanine green. Figure 3 shows the instantaneous indocyanine green extraction ratio in the KRB perfused lobe. The indocyanine green extraction ratio was nearly 100% on the rising portion of the [^{14}C]-dextran concentration vs. time curve. The extraction ratio then fell rapidly, indicative of reversible uptake of the dye by the lobe. In the plasma perfused lobes, the dye and dextran curves were superimposable and

Table 3 PGE_1 extraction in one lobe during plasma and KRB perfusion

Dose of PGE_1 (nmol)	*% extraction*		*% increase in PGE_1 extraction during KRB perfusion*
	Plasma perfusion	*KRB perfusion*	
0.06	89.3	95.3	6.7
189	75.9	91.0	19.9
565	27.2	44.0	61.8

no dye extration was detectable (that is, E was zero).

In previous studies, we observed that PGE_1 uptake by isolated cat lungs was greater in blood perfused than in KRB perfused lungs (Cozzini & Dawson, 1977b; Linehan *et al.*, 1981). In the present study, we found that when lobes were perfused with KRB as well as plasma, the PGE_1 extractions during KRB perfusion were greater than or equal to those during plasma perfusion (lobes no. 2, 7, 11 and 15). However, in these experiments, the PGE_1 extraction was so high even in the plasma perfused lobes that the experiments were not optimal for detecting increases in extraction. Therefore, to evaluate further the influence of perfusate composition on PGE_1 extraction, we studied one lobe in which the PGE_1 extraction was evaluated without dye when the dose of PGE_1 was varied to provided a wide range of extractions. Table 3 shows the influence of plasma and KRB perfusion on the PGE_1 extraction under these conditions. The extraction was always greater when the lobe was perfused with KRB. The difference between plasma and KRB was greatest when the dose of PGE_1 was high and the resulting extraction from plasma relatively low.

Another interesting observation was that when the indocyanine green was added to the reservoir in KRB perfused lungs, it caused a rather profound pulmonary vasoconstriction. This was never observed in plasma perfused lobes, nor with bromcresol green in plasma or KRB perfused lobes. The vasoconstriction was transient with peak arterial pressures increasing 2.5 to 3 times the baseline levels in about 40 s. The arterial pressure then returned to within 1.5 times the baseline levels within 4 min. We considered the possibility that the inhibitory effect of the dye might have been exacerbated by a reduction in the area of endothelial surface perfused, due to the vasoconstriction in the KRB perfused lungs. In lobe nos 4 and 15 (Table 2), we blocked the vasoconstrictor response by adding 25 $\mu g\,ml^{-1}$ papaverine hydrochloride to the perfusate before studying the PGE_1 extraction in the presence and absence of dye. We found that after eliminating the vasoconstrictor response, the indocyanine green still effectively inhibited the PGE_1 extraction, and conclude that the inhibitory effects of the dye on PGE_1 uptake predominate over any accompanying effects due to haemodynamics.

Discussion

The results of this study indicate that the inhibitory effects of indocyanine green and bromcresol green on PGE_1 extraction by the lungs was much greater in lungs perfused with a protein-free perfusate than in plasma perfused lungs. This difference is correlated with the observation that in plasma these dyes are mainly found bound to plasma proteins (Waldmann-Meyer & Schilling, 1956; Fox *et al.*, 1957; Cherrick *et al.*, 1960; Baker, 1966; Janecki & Krawcynski, 1970; Kawaski *et al.*, 1973; Deutschmann *et al.*, 1974; Kamisaka *et al.*, 1974) and suggests that those dye molecules which are bound to plasma protein do not contribute to the inhibition of PGE_1 uptake by the lungs. This is consistent with the observation of Hellewell & Pearson (1982), who found that sulphasalazine did not inhibit $PGF_{2\alpha}$ uptake by neonatal pig lungs when albumin was present in the lung perfusate.

Indocyanine green dye is of particular interest because of its clinical use in liver function studies (Cherrick *et al.*, 1960; Leevy *et al.*, 1967) and as a plasma tracer in indicator dilution studies (Fox *et al.*, 1957; Dawson *et al.*, 1975; Lewis *et al.*, 1982; Pitt *et al.*, 1982). In general, virtually 100% of the indocyanine green in plasma or serum samples has been recovered in the protein fractions obtained by ultracentrifugation, electrophoresis (Fox *et al.*, 1957; Cherrick *et al.*, 1960) or gel filtration (Janecki & Krawcynski, 1970; Kawasaki *et al.*, 1973), indicating that unbound indocyanine green has not been detectable in plasma by these methods. The indocyanine green binds to several plasma proteins, including prealbumin (Kamisaka *et al.*, 1974), albumin (Cherrick *et al.*, 1960; Kamisaka *et al.*, 1974), and various globulin fractions (Cherrick *et al.*, 1960; Baker, 1966; Kamisaka *et al.*, 1974), with the ratios being dependent on concentration (Kamisaka *et al.*, 1974), and species (Baker, 1966). The affinity of indocyanine green for plasma proteins is one of its important characteristics for indicator dilution studies, allowing it to be considered an intravascular indicator in all organs except the liver (Cherrick *et al.*, 1960). From the data shown in Figure 3, it is clear that in the absence of plasma protein the indocyanine green can be rapidly taken up by the lung. In addi-

tion, we found that in the absence of plasma protein, indocyanine green can cause pulmonary vasoconstriction. These observations, along with the fact that, as previously pointed out by Bakhle (1981), the free dye inhibits prostaglandin extraction, suggest that the high affinity of indocyanine green for plasma proteins, along with its relative lack of biological activity in the protein bound state may be important determinants of its lack of toxicity in clinical studies (Fox *et al.*, 1957; Fox & Wood, 1960; Leevy *et al.*, 1967).

Given the inhibitory effects of indocyanine green on prostaglandin extraction, it would be prudent to avoid the use of this dye in indicator dilution studies of prostaglandin uptake (Linehan & Dawson, 1979; Pitt *et al.*, 1982). However, based on the present study, it would appear that at clinically used concentrations in normal blood perfused dog lungs the binding of indocyanine green, to protein will prevent it from having much effect on prostaglandin extraction. Forder *et al.*,(1983) have observed that indocyanine green, at various concentrations including those used in the present study, depresses PGE_1 uptake by rabbit lungs *in vivo*. If one assumes that it is the unbound dye which is responsible for the inhibition, their study and ours taken together may imply species differences in the affinity of protein for the dye. This possibility is supported by the observation that species differences exist in the major indocyanine green binding protein fractions (Baker, 1966). One factor which might need to be considered when comparing different studies is that the physico-chemical properties of the dye and the rate of protein binding can be influenced by the solute composition of the indocyanine green dye solution infused (Baker, 1966; Tripp *et al.*, 1973; Landsman *et al.*, 1976). Depending on the location of the infusion relative to the lungs (i.e., time taken to reach the lungs) a reduced rate of protein binding might enhance the inhibitory effects of the dye.

The overall prostaglandin uptake by the lungs is dependent on several factors, one of which is particularly relevant here, namely; binding to plasma protein (Cozzini & Dawson, 1977; Hawkins *et al.*, 1977). In previous studies (Cozzini & Dawson, 1977b; Linehan *et al.*, 1981) we noted that the uptake of PGE_1 was increased when plasma proteins were absent from the perfusate. In the present study, we confirmed this observation in one lung using a wide range of injected doses of PGE_1 (Table 3). The effect of the plasma protein was most readily observable with higher doses of PGE_1 which produced extractions which were not already close to the maximum obtainable in the plasma perfused lobes. This observation was included in the present study to reiterate the importance of plasma proteins on PGE_1 uptake by the lungs. Thus, in general, our results appear to confirm the theory that PGE_1 uptake by the lungs and its inhibition by certain inhibitors can be dependent on whether or not plasma proteins are present in the perfusate. The protein composition of the perfusate is one of the factors which may need to be taken into account when examining pulmonary prostaglandin uptake and its inhibition.

This study was supported by the Veterans Administration and Research Grant HL-24349 from the National Heart, Lung and Blood Institute. The authors wish to thank Victoria Wagner-Weber and Jacqueline Owenby for their technical assistance and Mary McDermott for typing the manuscript.

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(*Received March 10, 1983.*
Revised July 26, 1983
Re-revised October 11, 1983)