Direct modulation of G-proteins by polyunsaturated fatty acids: a novel eicosanoid-independent regulatory mechanism in the developing lung

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Basal and fatty-acid-modulated G-protein function was studied in 1–3-day-pre-term, fetal guinea-pig, type II (fATII) pneumocyte apical membrane. Unstimulated (tonic) high-affinity GTPase activity (measured as $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ hydrolysis rate) was high and 77% pertussis toxin-insensitive. Alteration of this activity was used as a marker of G-protein regulation. Arachidonic acid (AA) showed a dose-dependent ($\text{IC}_{50} = 48 \pm 8 \mu\text{M}$) inhibition of activity at concentrations significantly below critical micellar concentrations; this effect was mimicked by other polyunsaturated fatty acids (IC_{50} for linoleic acid = $47 \pm 2 \mu\text{M}$; IC_{50} for oleic acid = $106 \pm 11 \mu\text{M}$). Saturated fatty acids showed no effect. The effect of AA on ouabain-insensitive ATPases in the same preparation was significantly lower, suggesting a specificity of

the GTPase modulation effect. AA modulation of GTPase activity was not attenuated by blocking eicosanoid metabolism with inhibitors of 5'-lipoxygenase, cyclo-oxygenase and P-450. In order to explore further the mechanism of AA-G-protein interaction, the effect of AA on the time course and equilibrium binding of $[\text{}^{35}\text{S}]\text{GTP}[\text{S}]$ to apical membrane was studied. Consistent with our GTPase assay data, AA inhibited binding with an IC_{50} value of $71 \pm 1 \mu\text{M}$; stearic acid did not mimic this effect. This is the first report of unsaturated-fatty-acid-specific modulation of lung G-protein function: since AA also up-regulates perinatal lung alveolar Na^+ transport, we suggest this lipid/G-protein switch helps maintain pulmonary fluid homeostasis around birth.

INTRODUCTION

Efficient and rapid lung-fluid absorption during the perinatal period [1] allows postnatal pulmonary ventilation and gas exchange. Failure of this process leads to alveolar fluid overload, an important factor in neonatal lung disease [2,3]. Lung-fluid absorption is Na^+ -driven, the alveolar Na^+ exit possibly occurring through the perinatal lung-alveolar type II pneumocyte apical membrane [4]. However, an inadequate understanding of lung Na^+ transport regulation at the cellular level leaves us deficient of a strategy for its clinical management.

Independently of metabolic conversion, micromolar concentrations of arachidonic acid (AA) and other fatty acids (FAs) regulate Na^+ , K^+ and Cl^- channels in a number of cell types, including squid giant axon, gastric smooth muscle, cardiac atrial, pulmonary arterial and aortic smooth muscle and airway epithelial cells; often the effect is specific to polyunsaturated fatty acids (PUFAs) [5]. In other systems, PUFAs control G-protein activity, with possible regulatory effects on cellular function [6–9]. We have recently reported FA- and G-protein-mediated regulation of conductive $^{22}\text{Na}^+$ transport in fetal alveolar type II (fATII) pneumocyte apical membrane vesicles [10] and in freshly isolated fATII cell Na^+ channels [11,12]; the FA effect is independent of its metabolic conversion to eicosanoids. This raises the possibility that the FA effect on lung alveolar Na^+ channels could be mediated via G-protein modulation. Thus demonstration of FA regulation of lung alveolar G-protein activity would constitute further evidence for a functional role of FA in the control of lung Na^+ transport. In this paper we report the kinetics of G-protein turnover and substrate binding in the term fATII pneumocyte, and its modulation by PUFAs. Finally, with neonatal PUFA deficiency now a likely clinical entity [13], we will speculate briefly on the possible implications of a

PUFA-specific G-protein-mediated role for the maintenance of lung alveolar Na^+ -linked fluid homeostasis around the time of birth.

EXPERIMENTAL

Materials

$[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (specific radioactivity 10 Ci/mmol) was purchased from Amersham (Amersham, Bucks., U.K.) and guanosine-5'-O-(3- $[\text{}^{35}\text{S}]\text{thio}$)triphosphate ($[\text{}^{35}\text{S}]\text{GTP}[\text{S}]$, specific radioactivity 1245 Ci/mmol) from DuPont–NEN. FAs were purchased from Cascade Biochem. The other chemicals used were purchased from Sigma.

Isolation and purification of apical membrane

The isolation and purification of type II pneumocyte apical membrane from fetal guinea-pig lung has been described in detail elsewhere [14]. Briefly, mature fetuses (1–3 days pre-term) were delivered by Caesarean section and the excised lung lobes homogenized in ice-cold homogenization medium, 250 mM sucrose/10 mM triethanolamine (TRA) (pH 7.6), containing 200 μg of DNase I, 2.6 mg of aprotinin A, 1.0 mg of leupeptin, 1.0 mg of pepstatin A and 17.5 mg of 4-(2-aminoethyl)-benzenesulphonyl fluoride per 100 ml of medium. Brush-border membrane fragments were separated and purified by differential centrifugation and divalent cation precipitation. The final pellet was resuspended in 250 mM mannitol/10 mM TRA (pH 7.6), the protein concentration measured by the method of Bradford [15], and aliquots stored in liquid N_2 until required. The purity of membrane preparation was assessed as described previously

Abbreviations used: AA, arachidonic acid; FA, fatty acid; PUFA, polyunsaturated fatty acid; TRA, triethanolamine; PTX, pertussis toxin; DTT, dithiothreitol; ETYA, eicosa-5,8,11,14-tetraynoic acid.

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Figure 1 High-affinity GTP hydrolysis is a function of reaction time and protein concentration

fATII apical membrane at protein concentrations of 0.1, 0.2 and 0.4 $\mu\text{g}/\mu\text{l}$ were added to the GTP assay mixture and the reaction terminated at the times indicated, as described in the text. Parallel tubes were incubated with 1 mM GTP to assess low-affinity GTP hydrolysis, which was subtracted from the total in order to calculate the high-affinity GTP hydrolysis shown in the Figure. The inset shows the linear relationship, at the 20 min time point, between rate of fATII apical membrane GTP hydrolysis and membrane protein concentration.

[14]. The apical membrane marker, alkaline phosphatase, was enriched > 19-fold, whereas mitochondrial, endoplasmic reticulum, lysosomal and basolateral marker enzymes showed no significant enrichment.

ADP-ribosylation of G-protein with pertussis toxin (PTX)

PTX (50 mg) was activated in a solution containing 115 mM NaCl, 10 mM TRA and 20 mM dithiothreitol (DTT) (pH 8.0) for 1 h at 37 °C. Apical membranes, resuspended in 105 mM NaCl/10 mM TRA, were ADP-ribosylated in the presence of 10 $\text{mg} \cdot \text{ml}^{-1}$ PTX for 1 h at 37 °C [16]. As this resulted in a final concentration of 200 μM DTT in the ribosylation mixture, all experiments using toxins employed a DTT control. The time course of ribosylation was followed with [^{32}P]NAD in the presence of PTX as described previously [16] but using the buffer modification above. Labelled proteins were separated on SDS/10% (w/v)-PAGE gels, dried and exposed to autoradiographic film. The time point of 1 h represented maximum labelling of a band at 41 kDa and was chosen for subsequent ribosylation experiments (results not shown).

Determination of G-protein-associated (high-affinity) GTPase enzyme activity

The final concentrations of assay mixtures (pH 7.5) in the reaction milieu were as follows: 1 mM 5'-adenylylimidodiphosphate, 1 mM ATP, 1 mM ouabain, 10 mM creatine phosphate, 2.5 units $\cdot \text{ml}^{-1}$ creatine phosphokinase, 100 mM NaCl, 5 mM MgCl_2 , 2 mM DTT, 100 μM EDTA, 10 mM TRA, 0.5 μM GTP, with [γ - ^{32}P]GTP and appropriate concentrations of FA, vehicle or 10^{-3} M unlabelled GTP. As the radiolabelled GTP needed to be added in trace amounts, it was necessary to make up the required concentration with unlabelled GTP. The final protein concentration used in the assays was 0.1 $\mu\text{g} \cdot \mu\text{l}^{-1}$ (see Figure 1). FAs were supplied dissolved in ethanol, and further

Figure 2 PUFAs inhibit high-affinity GTPase activity in fATII apical membrane

(a) Concentration-dependent inhibition of high-affinity GTPase activity by AA. FA was added to the reaction mixture 1 min before the start of the reaction. The reaction was activated by the addition of lung membrane preparation (IC_{50} 48 ± 8 μM , Hill coefficient 0.83 ± 0.12 , $n \geq 3$). (b) Concentration-dependent inhibition of high-affinity GTPase activity by linoleic (■) and oleic acid (▲) and the lack of inhibition with ETYA (▼) ($n \geq 3$). High-affinity GTPase activity was assessed as described in the text (linoleic acid, IC_{50} 47 ± 2 μM , Hill coefficient 1.34 ± 0.88 , $n \geq 3$; oleic acid, IC_{50} 106 ± 11 μM , Hill coefficient 0.99 ± 0.13 , $n \geq 3$; ETYA, IC_{50} 4.6 ± 0.9 mM, Hill coefficient 0.58 ± 0.29 , $n \geq 3$).

dilutions were made in 250 mM mannitol/10 mM TRA (pH 7.6); the concentration of ethanol in the final reaction milieu was maintained at 0.4% (v/v) (the control limb of the experiment also contained 0.4% ethanol; earlier experiments established that ethanol at this concentration did not affect G-protein activity). Except for the original time-course experiments, the reaction was terminated at 20 min by the addition of 900 μl per tube of 5% (w/v) activated charcoal slurry in 20 mM phosphoric acid (pH 2.3), as described by McKenzie [16]. This protein concentration and time point were chosen on the basis of data shown in Figure 1. Tubes were then centrifuged at 12000 g for 20 min to pellet the charcoal together with the unhydrolysed [γ - ^{32}P]GTP and the now-unlabelled GDP, whereas free $^{32}\text{P}_i$ remained in the supernatant. Radioactivity in the supernatant was assayed by liquid-scintillation counting. Low-affinity hydrolysis of [γ - ^{32}P]GTP (that not mediated by G-proteins) was assessed by parallel incubation of membrane with excess (1 mM)

GTP. The low-affinity activity was subtracted from the total to calculate the high-affinity GTPase activity.

Determination of [35 S]GTP[S] binding

Guanine nucleotide binding by G-protein α -subunits was quantified as described previously [17]. Briefly, the reaction was commenced by the addition of 5 μ g of membrane protein to a total assay volume of 250 μ l [37 °C, assay composition (pH 7.4) in the final milieu: 50 mM Tris/HCl, 1 mM EDTA, 5 mM MgCl_2 , 1 mM DTT, 100 mM NaCl, 0.2 units/ml adenosine deaminase, 1 % (w/v) BSA, 10 μ M GDP, 0.2 nM [35 S]GTP[S] and appropriate concentrations of FA, vehicle or 10^{-5} M unlabelled GTP[S]]. Non-specific [35 S]GTP[S] binding was assessed by parallel incubation of membrane with 10^{-5} M GTP[S]; this was subtracted from the total to calculate specific binding. Following the initial time course (see Figure 3a), equilibrium binding studies were carried out for 60 min incubations in the absence or presence of FAs. Reactions were terminated by the addition of ice-cold 50 mM Tris/HCl solution (pH 7.4) containing 5 mM MgCl_2 . Samples were kept ice-cold until filtration through glass-fibre filters (Whatman GF/B). Filters were dried and radioactivity determined by liquid-scintillation counting.

Statistics

Data are presented as means $\pm$ S.E.M. of three or more experiments. The Kruskal–Wallis one-way ANOVA (analysis of variance) test was used to compare multiple dependent variables. Differences were considered significant at $P < 0.05$. Dose–response data were fitted by an iterative fitting protocol using the Hill equation. Lines were fitted using the entire data sets and points plotted were means $\pm$ S.E.M. for clarity.

RESULTS

Characterization of G-protein-linked GTPase activity in lung apical membrane

Low-affinity GTPase activity (measured in the presence of 1 mM GTP) was small: approx. 10 % of the total. The rate of high-

affinity GTP hydrolysis was quasi-linear to 20 min (Figure 1), this point representing the best compromise between linearity and easy experimental manipulation, in line with others [16]. Basal high-affinity GTPase activity, a measure of unstimulated G-protein turnover, was 58 ± 7 pmol $\cdot$ min $^{-1}$ per mg of protein ($n = 28$). This value is surprisingly high for a preparation from non-excitable tissue [16] and suggests that lung maintains a large unstimulated G-protein turnover level. ADP-ribosylation of the membrane with PTX reduced basal high-affinity GTPase activity by 23 % (Table 4).

AA modulates high-affinity GTPase activity: the effect is mimicked by other PUFAs but not by eicosa-5,8,11,14-tetraynoic acid (ETYA)

The dose–response of AA on $G\alpha$ -GTPase activity was studied between 1 and 200 μ M (Figure 2a). Addition of 50 μ M AA produced an approx. 50 % inhibition of high-affinity GTPase activity ($IC_{50} = 48 \pm 8$ μ M; $n \geq 3$). Linoleate (18:2, $n = 6$; $IC_{50} = 47 \pm 2$ μ M; $n \geq 3$) resembled AA in its affinity for inhibition of GTPase activity. For oleate (18:1, $n = 9$; $IC_{50} = 106 \pm 11$ μ M; $n \geq 3$), the possession of only one double bond was apparently reflected in its diminished affinity. ETYA, with unsaturation in the form of four triple bonds and no double bonds, showed little effect on high-affinity GTPase activity ($IC_{50} = 4.6 \pm 0.9$ mM; $n \geq 3$) (Figure 2b). Ouabain-insensitive ATPase activity (known to be present in fATII apical membrane [18]) was significantly less affected (Table 1b), indicating that the FA interactions were likely to be specific to GTPase enzyme activity.

Saturated FAs are without effect on lung G-protein activity (Table 2)

The saturated FAs myristate (14:0), palmitate (16:0) and stearate (18:0), at similar concentrations (50 μ M), produced no significant change in $G\alpha$ -GTPase activity in the lung apical membrane. The difference between the unsaturated and the saturated FA effect on high-affinity GTPase activity was significant ($P < 0.01$).

Table 1 Comparison of AA modulation of fATII apical membrane ouabain-insensitive ATPase activity and high-affinity GTPase activity

Ouabain-insensitive ATPase activity was measured by inorganic phosphate production at 37 °C. The assay was initiated by the addition of membrane protein (4 μ g/ml) and terminated at 90 min (earlier experiments confirmed linearity at these concentration and time points) by the addition of 30 % (w/v) trichloroacetic acid and 1 % BSA. The reaction solution was held on ice for 20 min, centrifuged at 1500 g and 4 °C for 10 min and the supernatant assayed for inorganic phosphate using the Molybdate Blue method [18]. AA was added as appropriate, just before the start of the reaction. Ouabain (0.4 mM) was added as appropriate. (a) The time-course of ATPase activity confirms predominant ouabain-insensitivity. (b) AA (at its IC_{25} for high-affinity GTPase activity inhibition: 10 μ M) inhibits ouabain-insensitive ATPase activity by only 8 %; the difference is significant at $P < 0.01$. Values are means $\pm$ S.E.M.

(a)			
Time (min)	Total ATPase activity (pmol per mg of protein)	ATPase activity (+ 0.4 mM ouabain)	ATPase activity insensitive to ouabain (% of total)
0	0	0	0
30	30	24	80
75	42	35	83
120	57	46	81
165	68	59	87
(b)			
High-affinity GTPase activity inhibition		Ouabain-insensitive ATPase activity inhibition	
22.0 (6.7)		8.3 (0.5)	

Table 2 Effect of saturated and unsaturated FAs on GTPase activity

In a separate set of experiments, unsaturated FA (arachidonic, linoleic or oleic) or saturated FA (myristic, palmitic or stearic) was added to a final concentration of 50 μ M 1 min before the start of the reaction, which was activated by the addition of fATII membrane ($n \geq 3$). Whereas unsaturated FAs inhibited high-affinity GTPase activity as observed earlier, saturated FAs had no significant effect on this activity. Results are percentage inhibition of high-affinity GTPase activity in comparison with control. Results are means (S.E.M. in parentheses).

GTPase activity (% inhibition of control)					
Saturated FAs			Unsaturated FAs		
Myristic	Palmitic	Stearic	Linoleic	Oleic	Arachidonic
93 (8)	97 (7)	101 (4)	64 (3)	72 (3)	63 (2)

AA-specific G-protein regulation is not mediated via eicosanoid-generating pathways (Table 3)

In order to dissect the AA effect further into direct and metabolite-mediated components, the apical membrane preparation was treated with specific inhibitors of arachidonate metabolism, singly or in combination. The effect of the approximate IC_{50} of AA (50 μ M) was not significantly attenuated either by the cyclo-oxygenase inhibitor indomethacin (2 μ M) or by the 5'-lipoxygenase inhibitor nordihydroguaiaretic acid (2 μ M). Similarly, 50 μ M ETYA (a non-metabolizable AA analogue and a general blocker of all AA cascade enzymes at this concentration) had little effect on the AA-mediated inhibitory response, suggesting a direct effect of AA on G-protein activity.

AA (50 μ M) modulation of GTPase-activity ADP-ribosylation (Table 4)

AA-mediated modulation of high-affinity GTPase activity was relatively unaffected by mono-ADP ribosylation with PTX. Thus whereas PTX treatment blocks 23 % and AA (50 μ M) inhibits approx. 60 % of high-affinity GTPase activity, ADP-ribosylation followed by arachidonate treatment leads to partial additivity of the inhibitory effect to 50 % of control, suggesting that a significant component of the AA effect is via PTX-insensitive G-proteins. Although all experiments showed a similar trend, the differences between AA inhibition of GTPase activity in untreated and PTX-pretreated membranes is not significant at the $P < 0.05$ level.

Table 4 Effect of PTX pretreatment on basal and arachidonate-modulated high-affinity GTPase activity

Results are percentage inhibition of high-affinity GTPase activity in comparison with control. Results are means (S.E.M.). PTX pretreatment blocks approx. one-fifth of basal high-affinity GTPase activity, whereas 50 μ M AA causes approx. 50% inhibition. The effects of the two treatments were partially additive, which suggests independent regulatory pathways. Although experiments within each set showed a similar trend, the differences between the effect of arachidonate on control and PTX-pretreated membranes were not significant at the $P < 0.05$ level ($n = 5$).

GTPase activity (% inhibition of control)		
PTX pretreatment	AA	PTX pretreatment $\rightarrow$ AA
77 (5.2)	61 (11.0)	50 (13.2)

AA decreases [35 S]GTP[S] binding by fATII apical membrane (Figure 3)

To explore the mechanism underlying AA-induced inhibition of GTPase activity, the effect of AA on the ability of fATII apical membrane G-proteins to bind [35 S]GTP[S] was studied. The time course (Figure 3a) shows that AA reduces the rate and equilibrium position of [35 S]GTP[S] binding. Furthermore, addition of AA elicits a dose-dependent ($IC_{50} = 71 \pm 1 \mu$ M, $n \geq 3$) inhibition of [35 S]GTP[S] binding (Figure 3b). As with inhibition of high-affinity GTPase activity, this effect was not demonstrable with 50 μ M stearic acid ($IC_{50} = 5.1 \pm 2.9$ mM, $n \geq 3$).

DISCUSSION

This report significantly extends our earlier evidence in support of a physiological role for FAs in the control of lung alveolar Na^+ transport by identifying a possible mechanism for FA-mediated signal transduction in the type-II pneumocyte. In contrast with our findings that both saturated and unsaturated FAs stimulate alveolar epithelial Na^+ channels [10], only unsaturated FAs inhibit G-protein function in the same preparation. This is consistent with the presence of at least two co-existent FA-dependent regulatory pathways in the apical membrane of fATII cells: one acting via G-protein modulation and specific to FAs possessing double bonds, the other causing direct conformational change to membrane or Na^+ channels leading to their activation, which is independent of G-proteins and characteristic of both saturated and unsaturated FAs.

Both pathways have been identified recently in other systems. Whereas direct FA (10–100 μ M) control of ion channels has been

Table 3 Dissection of the pathway: use of eicosanoid synthesis and G-protein turnover block

Effect of blocking arachidonate metabolism on its inhibition of GTPase activity. All assays contained 50 μ M AA plus the inhibitors as appropriate: 100 μ M ETYA, 2 μ M indomethacin (INDO) and 2 μ M nordihydroguaiaretic acid (NDGA) ($n \geq 3$). None of the inhibitors of the enzymes of the arachidonate metabolic pathway significantly reversed the AA effect on GTPase activity. Results are percentage inhibition of high-affinity GTPase activity in comparison with control. Results are means (S.E.M.).

GTPase activity (% inhibition of control)				
Control	+ ETYA (100 μ M)	+ INDO (2 μ M)	+ NDGA (2 μ M)	+ INDO (2 μ M) + NDGA (2 μ M)
54 (5)	56 (3)	65 (5)	61 (3)	64 (1)

Figure 3 AA specifically inhibits $[^{35}\text{S}]\text{GTP[S]}$ binding to fATII membrane

(a) The effect of addition of AA on the time course of $[^{35}\text{S}]\text{GTP[S]}$ binding to fATII apical membrane. Reaction was commenced by the addition of 5 μg of fATII membrane to a total assay volume of 250 μl (37 $^{\circ}\text{C}$), with GTP[S] (100 μM) and/or AA (50 μM) added as indicated ($n \geq 3$). (b) The dose-dependency of AA inhibition of $[^{35}\text{S}]\text{GTP[S]}$ equilibrium binding. The IC_{50} value was very similar to that obtained for AA inhibition of GTPase activity (IC_{50} 71 ± 1 μM , Hill coefficient 0.51 ± 0.06 , $n \geq 3$). In contrast, an exemplar saturated FA (stearic) had no effect ($n \geq 3$).

well characterized [5], several recent reports have described selective PUFA interactions with signal-transducing proteins [6–9,19] and specific FA-activated receptors, apparently regulating cellular function in both prokaryotic and eukaryotic systems [20–22]. In addition to double bond specificity, an apparent relation between FA potency and the number of double bonds in the FA molecule has been noted by other workers, and is consistent with the data presented herein. For example, when bacterially synthesized c-Ha-ras protein (Ras) was incubated with GTPase-activating protein in the presence of saturated or unsaturated FAs, AA (20:4) at 50 $\mu\text{g}/\text{ml}$ (approx. 150 μM) blocked GTPase-activating protein activity by 88%. Linoleic acid (18:2) at similar concentrations was 33% inhibitory, whereas saturated FAs (palmitic, stearic) showed no effect [7]. Glick et al. [6] have observed a similar PUFA-specific (IC_{50} for AA = 60 μM ; IC_{50} values for PUFA with fewer double bonds were higher) down-regulation of binding of the non-hydrolysable GTP analogue, GTP[S], to the heterotrimeric G-protein G_z (not demonstrable for G_{i1} , G_{i3} , G_o , G_s ; not tested for $\text{G}_{q/11}$). We have studied the fATII apical membrane G-protein profile: at 1–3

days preterm, $\text{G}_{q/11}$ and $\text{G}_{i1,3}$ (but not G_z or G_s) are detectable by Western blotting [23]. We found the basal and PUFA-modulatable high-affinity GTPase activity in fATII membrane largely PTX-insensitive, suggesting a functionally more active role for $\text{G}_{q/11}$ in preference to other G-proteins in this membrane. The circumstantial evidence thus points towards $\text{G}_{q/11}$ as the primary candidate in G-protein regulation of lung alveolar epithelial function. Interestingly, phospholipase C acts as a GTPase-activating protein for $\text{G}_{q/11}$ [24], raising the possibility that AA could be acting via this lipase; this is consistent with a Hill coefficient of less than unity seen in Figure 5(b).

Alteration of high-affinity GTPase activity is a useful overall measure of G-protein function [25,26]. Because our target G-protein (possibly $\text{G}_{q/11}$) has a relatively rapid GTPase turnover [24], we have been able to utilize successfully this assay as our primary marker of PUFA effect on G-protein function. This assay is unlikely to be successful, though, when studying PUFA- G_z interactions (G_z -GTPase activity being very slow [27]), and Glick et al. [6] have appropriately used GTP[S] binding to G_z as their primary measure of effect. However, in order to strengthen further our hypothesis that the observed down-regulation of high-affinity GTPase activity was G-protein-mediated, we studied the time course of AA effect (at 50 μM , the approximate IC_{50} for high-affinity GTPase activity inhibition) together with the dose-response effect of AA on $[^{35}\text{S}]\text{GTP[S]}$ binding to fATII apical membrane. Furthermore, we used stearic acid as an exemplar saturated FA in order to study the specificity of the effect.

Our results of AA-induced down-regulation of $[^{35}\text{S}]\text{GTP[S]}$ binding conform to the observations of Glick et al. [6] and (given the general conservation of structure among G-proteins) point towards a similar mechanism of action, i.e. inactivation of the nucleotide-free form of $\text{G}\alpha$. However, we have no direct evidence for this mechanism and it remains likely that FAs are affecting GTP or GDP binding affinity (or both) at the guanosine nucleotide binding pocket of the α -subunit; our preliminary studies have indicated that affinity for GDP is unaffected by AA, but these observations remain to be substantiated. Our finding of significant AA effect ($\text{IC}_{25} \approx 10$ μM , $\text{IC}_{50} \approx 50$ μM) at levels much below the critical micellar concentration for AA (73 μM [6]) suggests that AA interaction with membrane proteins *in situ* is unlikely to be micelle-dependent, as suggested for AA-recombinant G_z interaction [6].

Other forms of physical perturbation of cellular components have been proposed as mechanisms for the dichotomy of effect between PUFAs and saturated FAs. Such perturbation could result from differences in aqueous solubilities of FAs, or from the ability of PUFAs to alter lipid acyl chain order [28]. It is of note, however, that the lipid permeability coefficients (K_p) of the mono- and poly-unsaturated FAs employed in our studies do not correlate well with their observed biological effect (K_p ratios of stearic acid/oleic acid/AA $\approx 26:5:1$ [28]). To define any non-specific components of the effect of AA on membrane nucleotidases, we tested the effect of AA on ouabain-insensitive ATPases (presumably the well-characterized fATII apical membrane K^+/H^+ -ATPase [29]). The small, non-significant inhibition (approx. 8% of control) of ouabain-insensitive ATPase activity probably represents the non-specific component of AA effect. At the same concentration, AA exerts a much larger and significant effect on high-affinity GTPase activity (22% inhibition), providing further evidence in favour of the physiological relevance of AA/G-protein interaction in the fATII apical membrane.

Although others have observed inhibition of G-protein activity with PUFAs (as referred to above) and other agonists [30], we are the first to note the apparent association of such inhibition

with up-regulation of cellular function [10]. G-protein-mediated signal transduction is not simply a linear process, but rather involves significant cross-talk between different G-protein-coupled pathways and other signal-transduction systems [31]. Set against this complexity of opposing (stimulatory and inhibitory) G-protein interactions known to regulate other aspects of ATII cell function [32], we would like to speculate briefly on mechanisms that could explain this observed association between G-protein inhibition and stimulation of Na^+ transport in fATII apical membrane. If Na^+ channels were under tonic G-protein inhibition, inactivation of $G\alpha$ will reduce such control, and this could lead to augmentation of Na^+ transport. Alternatively, $G\alpha$ inactivation could hinder re-association of the heterotrimeric G-protein, facilitating cellular function up-regulated by the $\beta\gamma$ dimer. Mechanisms by which a PUFA effect on G-proteins could be transiently inhibitory (thus allowing participation in a feed-back regulatory process) have recently been discussed [6].

Given the well-established relationship between alveolar fluid overload and neonatal respiratory disease [2], the identification of a possible link between AA-mediated signal transduction and alveolar Na^+ transport up-regulation at birth raises the important clinical question: is perinatal PUFA deficiency (prevalent in early infancy [13]) an unidentified aetiological factor for respiratory morbidity? Antenatal dietary PUFA deficiency in mice directly reduces pup-lung phospholipid AA content [33], whereas dietary PUFA deficiency in infancy (in standard formula-fed compared with breast-fed infants) is associated with greater respiratory morbidity [34–36]. Surprisingly, this difference in morbidity persists for many years beyond the time when PUFA intake differed significantly between the two cohorts [35,36]. Recent epidemiological evidence favours this hypothesis of early 'programming' contributing to disease in later life [37]. Could PUFA homeostasis play a role in the perinatal lung, influencing the pattern of future respiratory disease? A clearer understanding of PUFA-mediated cellular regulation will allow us to explore this putative link between diet and lung disease.

We thank Alan Monaghan, Mark Clunes, Debbie Baines, Gregor Fyfe, Gordon MacGregor, Gordon Tennant and Craig Gould for laboratory and statistical assistance, and the Wellcome (Grant Nos. 042224/Z/94/Z and 039124/Z/4A) and Buttle Trusts for financial support.

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