Introduction
Airway smooth muscle controls airway calibre and therefore determines alveolar ventilation. Disease states such as asthma are characterized by an increased muscle tone and airways resistance, which are due, at least in part, to changes in the response of airway smooth muscle to endogenously generated stimuli [11]. Clearly, an understanding of the physiological and pathophysiological regulation of smooth muscle tone is central to the development of novel therapeutic approaches for the treatment of airway hyper-reactivity.

In this brief review, we attempt to summarize some of the progress that has been made recently in our understanding of phosphoinositide signalling in airway smooth muscle, with a particular emphasis on how changes in cyclic nucleotides and membrane potential may modulate the phosphoinositide cycle.

Contractile agonists and phosphoinositide turnover
Contraction of airway smooth muscle can be provoked by a variety of agents (termed spasmogens) that interact with cell-surface receptors to effect pharmacomechanical coupling [2, 3]. Agents may be released endogenously from neurons, may be blood-borne or may be released from pro-inflammatory cells that can infiltrate the airways under certain circumstances [4]. Although many spasmogenic agents cause significant depolarization of airway smooth muscle, it is generally considered that it is their ability to stimulate phosphoinositide-specific phospholipase C (PI-PLC), via a guanine-nucleotide-binding protein (G-protein), and cause the generation of the second messengers inositol 1,4,5-trisphosphate \(\text{Ins}(1,4,5)P_3\) and diacylglycerol (DAG), which underlies their contractile action [2, 3].

Considerable progress has been made in characterizing the array of receptors that can stimulate PI-PLC activity in this tissue. Thus, acetylcholine (via \(\text{M}_3\)) [5], histamine (via \(\text{H}_1\)) [6], bradykinin (via \(\text{B}_2\)) [7], endothelin [8] and a variety of other agents including neuropeptides (for example, tachykinins [9]) and mediators (for example, leukotrienes [10] and thromboxanes [11]) can all evoke an increase in phosphoinositide turnover in preparations of airway smooth muscle. Although the majority of these receptors appear to recruit G-proteins of the pertussis-toxin-insensitive \(\text{G}_q\) family selectively to stimulate PI-PLC [3, 12, 13], there is evidence that some agents (for example, leukotriene \(\text{D}_4\) [10]) act via pertussis-toxin-sensitive transduction mechanisms.

In some cases, most notably for \(\text{M}_1\)-muscarinic and \(\text{H}_1\)-histaminergic receptor activation, the time-course, concentration-dependency and pattern of inositol (poly)phosphate generation has been investigated in some detail. Muscarinic receptor agonists or histamine cause a sustained activation of

Abbreviations used: DAG, diacylglycerol; PI-PLC, phosphoinositide-specific phospholipase C.
PI-PLC activity, such that, in the presence of a concentration of Li⁺ that is sufficient to completely prevent inositol monophosphate hydrolysis, linear accumulations of total [³H]inositol phosphates can be observed for at least 30 min in myo-[³H]inositol-labelled airway smooth muscle preparations [14-16]. Although the ability of these agonists to cause a sustained activation of phosphoinositide turnover is reflected in the time-course of changes in membrane inositol phospholipids [17, 18], only rapid, transient increases in the concentration of Ins(1,4,5)P₃ have been observed [16, 18-20], with cellular levels of this second messenger returning to pre-stimulation levels within 60 s of agonist addition. Therefore, although peak Ins(1,4,5)P₃ accumulation precedes initiation of contraction temporally, and is consistent with Ins(1,4,5)P₃ interacting with a specific receptor [21] to induce Ca²⁺ mobilization from organellar stores being sufficient and necessary for contraction [22, 23], the role of increased phosphoinositide turnover in the maintenance of contraction is less clear. A widely held view is that translocation and activation of protein kinase C by DAG and Ca²⁺ are important in maintaining smooth muscle contraction [24-26], with sustained activation of phosphoinositide turnover providing one possible source of DAG. Furthermore, recent studies have provided direct evidence that a number of contractile agonists cause sustained increases in Ca²⁺ entry by activating receptor-operated Ca²⁺ channels [27, 28] and positively modulating voltage-operated Ca²⁺ channels [29, 30], possibly via a second messenger (DAG/protein kinase C [31]) route.

Thus, agonist-stimulated phosphoinositide turnover provides the second messenger that is responsible for initiating pharmacomechanical coupling, and the sustained activation of this signalling pathway may be important in providing DAG during the maintained tonic phase of contraction.

**Cyclic-nucleotide-elevating agents and the phosphoinositide cycle**

It has been established for some time that elevations of cyclic AMP or cyclic GMP concentration in airway smooth muscle can cause relaxation of spontaneous and spasmogen-supported tone [32]. Indeed, the use of β-adrenoceptor agonists to cause bronchodilatation has been a mainstay of asthma therapy for several years [33]. There appears to be no simple correlation between changes in airway smooth muscle cyclic nucleotide levels and relaxation. The observed relaxation has been shown to depend on a number of factors, which include the degree of initial spasmogen-supported tone, the spasmogen used to induce the tone and the concentration and type of cyclic-nucleotide-elevating agent used to cause relaxation [34-37]. A common, although not universal [38], observation is that cyclic-nucleotide-elevating agents exhibit a decreased potency for relaxation against tone induced by muscarinic receptor agonists compared with that induced by a number of other contractile agonists (see below).

The mechanism by which cyclic-nucleotide-elevating agents cause relaxation of airway smooth muscle has been investigated extensively. One key observation that was made only relatively recently was that, contrary to an earlier report [39], cyclic-AMP-elevating agents could inhibit spasmogen-stimulated phosphoinositide turnover in airway smooth muscle preparations [40, 41]. A striking feature of the results reported by Hall and Hill, and Madison and Brown was that in bovine [40] and canine [41] tracheal smooth muscle preparations β-adrenoceptor agonists dramatically and concentration-dependently inhibited (by up to 50-75%) histamine-stimulated total [³H]inositol phosphate accumulation, but were completely ineffective when carbachol or methacholine were used to elicit phosphoinositide turnover. The studies of Madison and Brown [41] and subsequent work by others [42-44] demonstrated that other cyclic-AMP-elevating strategies (for example, forskolin, phosphodiesterase inhibition) had similar inhibitory effects. In addition, an inhibitory effect on [³H]inositol phosphate accumulation could be observed when a non-selective phosphodiesterase inhibitor, isobutylmethylxanthine, was used against sub-maximally effective concentrations of muscarinic agonist [43, 44].

The differential effects of cyclic-AMP-elevating agents on histamine- as against muscarinic cholinoreceptor-stimulated phosphoinositide turnover bear a striking resemblance to those reported previously in contractile studies. One possible explanation that has been proposed relates to the large 'receptor reserve' that exists for full muscarinic agonists [45-47]. Thus, in bovine tracheal smooth muscle, stimulation of [³H]inositol phosphate accumulation to 3.5% of that elicited by a maximally effective concentration of a full-muscarinic agonist is sufficient to cause a contraction that is 50% of the maximal response [48]. However, [³H]inositol phosphate accumulation stimulated by partial muscarinic agonists also exhibits differential sensitivities to cyclic-AMP-elevating strategies [16, 1993].
which suggests that 'receptor reserve' cannot fully explain the observed effects.

Airway smooth muscle, in common with a variety of other smooth muscle types, expresses a mixed population of muscarinic receptor subtypes. In addition to the M<sub>1</sub>-muscarinic receptor population that is responsible for stimulating phosphoinositide turnover, M<sub>2</sub>-muscarinic receptors are also present [5, 12, 46, 49, 50]; indeed, M<sub>2</sub>-sites often constitute the predominant muscarinic receptor subtype [12, 51]. A number of studies have demonstrated that M<sub>2</sub>-receptor stimulation can inhibit adenylyl cyclase activity in airway smooth muscle membranes [52], and decrease β-adrenoceptor- or forskolin-stimulated cyclic AMP accumulation in tracheal smooth muscle strips [51], slices (Figure 1) and enzymically dissociated cells [47]. Clearly, the presence of M<sub>2</sub>-muscarinic receptors may provide a possible explanation for why muscarinic receptor-induced contraction and/or phosphoinositide turnover shows a decreased susceptibility to inhibition by cyclic-AMP-elevating agents.

A number of experimental approaches have been used in an attempt to establish the relative roles of M<sub>2</sub>- as against M<sub>1</sub>-mediated effects in airway smooth muscle. These include exploitation of the fact that M<sub>2</sub>-mediated effects (but not M<sub>1</sub>-mediated effects) are pertussis-toxin-sensitive [12, 51], and the use of muscarinic agonists that exhibit M<sub>2</sub>/M<sub>3</sub> selectivity [5, 12, 46, 47]. Although conflicting

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**Figure 1**

Evidence that M<sub>2</sub>-muscarinic cholinoreceptors are coupled functionally to the inhibition of adenylyl cyclase in bovine tracheal smooth muscle.

Cross-chopped smooth muscle slices (300 μm x 300 μm) were prepared and incubated as described in [16]. Where indicated, methoctramine was added 15 min before carbachol at the concentrations indicated. After a 5 min pre-exposure period, forskolin (10 μM) was added and incubations terminated after 5 min. Values are shown as means ± S.E.M. for three separate experiments performed in triplicate. IC<sub>50</sub> values were determined for each of the carbachol concentration-response curves, and the calculated concentration ratios presented as a Schild plot, which yielded a pA<sub>2</sub> value of 7.72 (and Schild slope factor of 1.09).
findings have arisen (see [38, 53]), there is a growing body of evidence for a functional antagonistic action of M₂-receptor activation opposing β-adrenoceptor-induced relaxation of muscarinic-agonist-supported tone [50, 54]. Furthermore, a number of mechanisms by which co-stimulation of M₂/M₁-receptor populations results in a decreased susceptibility to inhibition by cyclic-AMP-elevating agents have been proposed. These include, in addition to the demonstrable adenylyl cyclase inhibitory action of M₂-receptor activation, a G₁-dependent functional inactivation of G₃ activity [55], and an inhibitory effect of methacholine (but not histamine) on cyclic-AMP-dependent protein kinase activity [35, 36] in airway smooth muscle preparations. However, conclusive evidence that M₂-muscarinic receptor stimulation accounts for, or contributes to, the resistance of muscarinic-receptor-stimulated phosphoinositide turnover to inhibition by cyclic-AMP-elevating agents has not yet been forthcoming.

Like the effects of cyclic AMP, agents that increase cyclic GMP concentration cause relaxation of airway smooth muscle [32, 35, 56, 57]. Perhaps the first attempt to assess whether cyclic-GMP-elevating agents could inhibit spasmogen-stimulated phosphoinositide turnover in airway smooth muscle came to a momentous conclusion [58]. Langlands and colleagues [58] reported that increasing cyclic GMP concentration in guinea pig tracheal rings using a cyclic-GMP-specific phosphodiesterase inhibitor (zaprinast) had no significant effect on the rate and extent of contraction induced by histamine or methacholine, but could inhibit Ins(1,4,5)P₃ accumulation stimulated by either spasmogen completely. Such a finding challenged the widely accepted second messenger role for Ins(1,4,5)P₃ in pharmacomechanical coupling in airway smooth muscle. An obvious criticism of this work is that smooth muscle is a relatively minor constituent of the preparation employed, therefore studies were designed to reproduce the experimental conditions of Langlands et al. faithfully, but using an airway preparation containing a much higher proportion of smooth muscle (>95%). Chilvers et al. [57] could find no evidence for an inhibitory effect of zaprinast either on total [³H]inositol phosphate accumulation or on Ins(1,4,5)P₃ accumulation stimulated by methacholine, confirming a previous study [43]. Figure 2 illustrates our own complementary data that demonstrate that sodium nitroprusside, an agent that dramatically increases cyclic GMP accumulation via activation of soluble guanylyl cyclase, does not significantly inhibit histamine-stimulated [³H]inositol phosphate accumulation in bovine tracheal smooth muscle. Therefore, although a species difference between guinea pig-derived and bovine-derived airway smooth muscle has yet to be excluded, we are confident that the observations of Langlands et al. [58] do not represent a general phenomenon.

**Membrane potential and the phosphoinositide cycle**

Agents that increase the probability of K⁺-channel opening cause membrane hyperpolarization of airway smooth muscle and promote relaxation.

**Figure 2**

Inhibitory effect of isoprenaline, BRL 38227 and sodium nitroprusside on histamine-stimulated [³H]inositol phosphate accumulation in bovine tracheal smooth muscle

Cross-chopped smooth muscle slices (300 µm x 300 µm) were prepared and incubated as described in [16]. Slices were incubated with [³H]inositol (1 μCi/ml) for 60 min in the presence of 1 μM carbachol to facilitate inositol phospholipid labelling [14]. After extensive washing, slices were incubated with 5 mM LiCl in the continued presence of [³H]inositol. The concentrations of isoprenaline, BRL 38227 or sodium nitroprusside indicated were added 15 min before the addition of histamine (100 µM) after which incubations were continued for 30 min. Under these conditions [³H]inositol phosphate accumulation were basal, 305 ± 39, histamine-stimulated, 10'009 ± 977 d.p.m./mg of protein (pooled data for six experiments). Values are shown as a fraction of the histamine-stimulated [³H]inositol phosphate accumulation and are in all cases means ± S.E.M for three separate experiments performed in triplicate. In parallel experiments, isoprenaline (10 µM) caused a 4-8-fold increase in cyclic AMP concentration, and sodium nitroprusside (100 µM) an 8-12-fold increase in cyclic GMP concentration. BRL 38227 had no effect on either cyclic nucleotide.
Furthermore, the ability of K⁺-channel openers to inhibit agonist-induced bronchospasm in vivo effectively [62] has resulted in considerable interest in the therapeutic potential of this class of agent in the treatment of airway hyperresponsiveness [61].

We have provided evidence recently that BRL 38227, the active (−) (3S,4R) enantiomer of the prototypic K⁺-channel opener, cromakalim [63], can affect agonist-stimulated phosphoinositide turnover in a manner superficially resembling that observed previously for cyclic-AMP-elevating agents [16, 64]. Thus, BRL 38227 inhibited histamine-stimulated [³H]inositol phosphate accumulation dramatically, whilst also inhibiting stimulation of this response by low concentrations of carbachol [16, 64]. A concentration of BRL 38227 that caused maximal inhibition of agonist-stimulated phosphoinositide turnover had no effect on airway smooth muscle cyclic AMP or cyclic GMP levels [60, 64], suggesting that cyclic nucleotides are not involved in the inhibitory effect. In addition, the effects of BRL 38227 were antagonized by glibenclamide [16], suggesting ATP-sensitive K⁺-channels (KATP) [65] may be important in mediating the inhibitory effects of BRL 38227. That membrane potential may modulate the histamine-stimulated phosphoinositide response was supported by the fact that the inhibitory effect of BRL 38227 could be decreased, and ultimately prevented, by depolarizing airway smooth muscle by increasing the extracellular concentration of K⁺ [16, 66].

It should be noted that, although the initial transient increase in Ins(1,4,5)P₃ accumulation stimulated by histamine is unaffected by decreasing extracellular Ca²⁺ concentration (R. A. J. Challiss and R. Mistry, unpublished results), [³H]inositol phosphate accumulation measured over longer periods of agonist challenge (30 min) is decreased dramatically by lowering extracellular Ca²⁺ concentration (Figure 3). Similar effects can be observed when calcium channel antagonists are used to block Ca²⁺ entry [16]. These results, together with other data obtained using patch-clamp techniques [29, 30], suggest that spasmogen-stimulated Ca²⁺ entry via voltage-operated Ca²⁺ channels is important for the maintenance of the sustained [³H]inositol phosphate generation caused by these agents. Thus, either direct inhibition of the Ca²⁺ entry pathway using L-type Ca²⁺-channel antagonists [16], or decreasing the probability that voltage-operated Ca²⁺-channels will be open by membrane hyperpolarization [16] result in a profound inhibition of histamine-stimulated [³H]inositol phosphate accumulation.

With respect to the mode of action of K⁺-channel openers, it is interesting to note that β-adrenoceptor agonists also cause membrane hyperpolarization of airway smooth muscle [67]. The evidence available so far suggests that the hyperpolarizing effects of β-adrenoceptor agonists are mediated via a distinct class of K⁺-channel, high conductance Ca²⁺-activated K⁺-channels (KCa, [65]), both via G-protein [68] and via cyclic-AMP-dependent protein-kinase-mediated [69] mechanisms. Our own recent studies suggest that isoprenaline inhibition of histamine-stimulated [³H]inositol phosphate accumulation may rely, at least in part, on the hyperpolarizing effects of this agent in airway smooth muscle. Thus, inhibition by isoprenaline can be attenuated dramatically by increasing extracellular K⁺ concentration [66], and...
histamine-stimulated [\(^{3}H\)]inositol phosphate accumulation can be inhibited via a glibenclamide-insensitive [66], but charybotoxin-sensitive (D. Adams and R. A. J. Challis, unpublished results) mechanism. Furthermore, the inhibitory effects of isoprenaline and BRL 38227 appear to be additive, suggesting that increased open probabilities of \(K\text{v}_{\text{ATP}}\) result in a more marked attenuation of the agonist-stimulated phosphoinositide response [66].

**Concluding remarks**

The data presented above suggest that \(\beta\)-adrenoceptor agonists and \(K\text{v}_{\text{ATP}}\)-channel openers exert an inhibitory effect on histamine-stimulated phosphoinositide turnover through their common ability to cause membrane hyperpolarization and to limit \(Ca^{2+}\)-entry via L-type channels. Spasmogens can activate multiple pathways of \(Ca^{2+}\)-entry in airway smooth muscle, and it has yet to be determined whether \(\beta\)-adrenoceptor agonists or \(K\text{v}_{\text{ATP}}\)-channel openers have any influence on the receptor-operated \(Ca^{2+}\)-entry pathway described by Kotlikoff and colleagues [27, 28].

The absolute or relative resistance of muscarinic-receptor-stimulated phosphoinositide turnover to inhibition by \(\beta\)-adrenoceptor agonists or \(K\text{v}_{\text{ATP}}\)-channel openers respectively remains to be explained fully. That the sustained phase of muscarinic receptor-stimulated phosphoinositide turnover is less dependent on \(Ca^{2+}\)-influx, together with the recent evidence that \(K\text{v}_{\text{ATP}}\) channels are inhibited by muscarinic receptor activation via a pertussis-toxin-sensitive pathway [68, 70] may provide at least a partial explanation.

In contrast to the recently described effects of membrane hyperpolarization on agonist-stimulated phosphoinositide turnover in vascular smooth muscle [71], it should be noted that \(\beta\)-adrenoceptor agonists and \(K\text{v}_{\text{ATP}}\)-channel openers do not affect the initial transient increase in \(\text{Ins}(1,4,5)P_{3}\) mass observed in bovine tracheal smooth muscle after histamine challenge (R. A. J. Challis and R. Mistry, unpublished results) [16]. This may explain why a short 'lag-phase' is observed in time-course experiments before significant inhibitory effects of \(\beta\)-adrenoceptor agonists [40] and \(K\text{v}_{\text{ATP}}\)-channel openers [16] on histamine-stimulated [\(^{3}H\)]inositol phosphate accumulations are seen, and why cross-talk between these two signalling pathways has sometimes not been reported [72].

The functional consequences of an inhibition of spasmogen-stimulated phosphoinositide turnover that becomes apparent during the sustained phase of airway smooth muscle contraction have yet to be established. Unlike spasmogen-stimulated increases in \(\text{Ins}(1,4,5)P_{3}\), the elevation of DAG appears to be sustained throughout the period of spasmogen challenge [17]. Thus, it is now important to establish whether the effects of the inhibitory agents described here exert an action at the level of the second messenger in the pathway DAG, before the true importance of such cross-talk mechanisms can be ascertained.

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The ability of 5-hydroxytryptamine (5-HT) to augment contractions evoked by other agents (and vice versa) in vascular smooth muscle has been studied for many years (see [1, 2] for reviews). The abundance of the data available contrasts starkly with the lack of knowledge of the mechanisms underlying such amplification phenomena. Our own interest in 5-HT stems from not only the proposed involvement of this amine in cardiovascular disease involving platelet aggregation, but also from the more recent knowledge that both 5-HT1 and 5-HT2 receptors mediate contraction of vascular smooth muscle. Thus, there is a multitude of possible interactions between the various factors released from aggregating platelets. We have examined the interactions between the platelet-derived products 5-HT and thromboxane A2 (TxA2), which are found in elevated concentrations in the plasma of patients with cardiovascular disease [3, 4].

Interactions between 5-HT and TxA2 were studied in the rabbit femoral artery [5, 6]. Briefly, vascular ring segments were suspended in organ baths filled with Krebs' saline for recording isometric force changes. As this tissue possesses both 5-HT1 and 5-HT2 receptors, one of these has to be eliminated to examine exclusively the interaction between 5-HT1 or 5-HT2 receptors and TxA2 receptors. 5-HT1 receptors were isolated by including the 5-HT2 receptor antagonist spiperone (0.3 μM) in the saline. 5-HT2 receptors were isolated by irreversibly alkylating 5-HT2 receptors using benextramine tetrahydrochloride (3 μM for 30 min). Interactions were examined by inducing contraction corresponding to 10%, 30% or 50% of the tissue maximum with one agonist (A10, A30 and A50) and, at steady state, a concentration-effect curve constructed to a second agonist.

**Interactions between 5-HT1 and TxA2 receptor-mediated responses**

Concentration-effect curves to the selective 5-HT1 receptor agonist ± α-methyl-5-hydroxytryptamine (α-Me-5-HT) were obtained in the presence and in the absence of the stable TxA2-mimetic U46619 (Figure 1a). Responses to α-Me-5-HT (control midpoint location [pA50] = 6.82 ± 0.04) were displaced significantly to the left in the presence of U46619, the maximum change in midpoint location occurred when the tissues were contracted with U46619 to 30% of their maximum (pA50 = 7.24 ± 0.09). At the highest concentration of U46619 (A50) the α-Me-5-HT midpoint location moved back towards that of the control (pA50 = 7.16 ± 0.07). There was no significant increase in the maximum response to α-Me-5-HT at any concentration of U46619. These data can be illustrated further in the form of histograms showing the effects of different concentrations of α-Me-5-HT and U46619, alone and in combination (Figure 1b). This shows that, at lower concentrations only, the effect of these two agents when applied in combination is greater than the simple sum of their individual effects.

The interaction between agonists acting at 5-HT1 and at TxA2 receptors are consistent with the expectations of threshold synergy [5]. A theoretical model of threshold synergy [7] proposes that if the transduction pathways of different receptors merge, then the model can be used to establish conditions under which agonist-agonist interactions

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**Abbreviations used:** A, agonist; α-Me-5-HT, (±)-α-methyl-5-hydroxytryptamine; 5-HT, 5-hydroxytryptamine; NPY, neuropeptide Y; [pA50], midpoint location of agonist concentration-effect curves; PIP2, phosphatidylinositol 4,5-bisphosphate; TxA2, thromboxane A2.

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