Eur Respir J 2006; 27: 1286–1306
DOI: 10.1183/09031936.06.00112605


**SERIES "SIGNALLING AND TRANSCRIPTIONAL REGULATION IN INFLAMMATORY AND IMMUNE CELLS: IMPORTANCE IN LUNG BIOLOGY AND DISEASE"**
**Edited by K.F. Chung and I.M. Adcock**
**Number 5 in this Series**

# Beyond the dogma: novel $\beta_2$-adrenoceptor signalling in the airways

**M.A. Giembycz* and R. Newton#**

**ABSTRACT: $\beta_2$-Adrenoceptor agonists evoke rapid bronchodilatation and are the mainstay of the treatment of asthma symptoms worldwide. The mechanism of action of this class of compounds is believed to involve the stimulation of adenylyl cyclase and subsequent activation of the cyclic adenosine monosphosphate (cAMP)/cAMP-dependent protein kinase cascade.**

**This classical model of $\beta_2$-adrenoceptor-mediated signal transduction is deeply entrenched, but there is compelling evidence that agonism of $\beta_2$-adrenoceptors can lead to the activation of multiple effector pathways, which now compels researchers in academia and the pharmaceutical industry alike to think beyond the traditional dogma. Therefore, the regulation by $\beta_2$-adrenoceptor agonists of responses, including airways smooth muscle tone and the secretory capacity of the epithelium and pro-inflammatory/immune cells, may be highly complex, involving both cAMP-dependent and -independent mechanisms that, in many cases, may act in concert.**

**In this article, the current status of $\beta_2$-adrenoceptor-mediated signalling in the airways is reviewed in the context of understanding mechanisms that may underlie both the beneficial and detrimental effects of these drugs in asthma symptom management.**

**KEYWORDS: Airways smooth muscle, asthma, $\beta_2$-adrenoceptor agonists, cyclic adenosine monosphosphate signalling**

Short-acting $\beta_2$-adrenoceptor agonists are the most effective and safest bronchodilators currently available, and are the drugs of choice for the rapid alleviation of bronchoconstriction that occurs in asthma. This class of compounds relaxes airways smooth muscle (ASM) irrespective of the constrictor stimulus and are well tolerated by most patient groups [1–3]. The introduction, in the early 1990s, of long-acting $\beta_2$-adrenoceptor agonists and their widespread inclusion into primary healthcare regimens (*e.g.* 2003 British Thoracic Society guidelines [4]), as well as the clinical development of other cyclic adenosine monosphosphate (cAMP)-elevating compounds, such as phosphodiesterase (PDE) 4 inhibitors, has led to the realisation that cAMP elevation in relevant cells may also provide additional benefits, including possible anti-inflammatory activity [5]. Furthermore, the widely reported beneficial effects in asthma of long-acting $\beta_2$-adrenoceptor agonists in combination with inhaled glucocorticoids has led to renewed interest in the molecular basis underlying this enhanced therapeutic effect [6]. Despite improved asthma treatment options, adverse effects are not reported infrequently and increased numbers of asthma-related deaths associated with high-level use of certain agonists are well documented [7]. Although these adverse clinical events may be due to receptor or even whole pathway desensitisation [8], studies since the late 1990s suggest that promiscuous coupling

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Received:
September 27 2005
Accepted after revision:
January 25 2006

SUPPORT STATEMENT
R. Newton and M.A. Giembycz are Alberta Heritage Foundation for Medical Research (Edmonton, AB, Canada) Scholar and Senior Scholar, respectively, and are funded by the Canadian Institutes for Health Research (Ottawa, ON, Canada) and the Alberta Lung Foundation (Edmonton, AB, Canada).

**Previous articles in this series: No. 1:** Fan J, Heller NM, Gorospe M, Atasoy U, Stellato C. The role of post-transcriptional regulation in chemokine gene expression in inflammation and allergy. *Eur Respir J* 2005; 26: 933–947. **No. 2:** Georas SN, Guo J, De Fanis U, Casolaro V. T-helper cell type-2 regulation in allergic disease. *Eur Respir J* 2005; 26: 1119–1137. **No. 3:** Boxall C, Holgate ST, Davies DE. The contribution of transforming growth factor-β and epidermal growth factor signalling to airway remodelling in chronic asthma. *Eur Respir J* 2006; 27: 208–229. **No. 4:** Barnes PJ. Corticosteroid effects on cell signalling. *Eur Respir J* 2006; 27: 413–426.

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

of the $\beta_2$-adrenoceptor to effectors other than the traditional cAMP/cAMP-dependent protein kinase (PK)A cascade may also be involved [9]. More fundamentally, the molecular mechanisms by which $\beta_2$-adrenoceptor agonists cause ASM to relax are still not completely defined. It is likely that areas of deficiency in the understanding of $\beta_2$-adrenoceptor function and action will provide explanations for many of these observations and continued research may lead to improved therapeutic approaches. In the present article, $\beta_2$-adrenoceptor signalling in the airways is reviewed and related, where possible, to the clinical effects of selective agonists.

## THE DOGMA AND BEYOND

It is well established that the $\beta_2$-adrenoceptor can couple *via* the heterotrimeric stimulatory G-protein (Gs) to adenylyl cyclase (AC), thereby enhancing the rate of cAMP biosynthesis. One primary consequence of this signalling is the activation of PKA, which, according to conventional dogma, mediates the ability of cAMP to cause smooth muscles to relax by a variety of complementary mechanisms (fig. 1a) [2, 3, 10–14]. However, incremental advances in the understanding of $\beta_2$-adrenoceptor-mediated signal transduction since the mid-1990s have shown that this deceptively simple signalling cascade is, in fact, highly complex, involving multiple variants of AC, PKA and PDE, as well as scaffolds, such as PKA anchoring proteins (AKAPs), which bind PKA and play a role in targeting the kinase to specific intracellular locations [15–19]. In addition, data obtained from studies using inhibitors of AC and PKA suggest the existence of cAMP- and PKA-independent mechanisms of ASM relaxation evoked by $\beta_2$-adrenoceptor agonists (fig. 1b–e) [20–22]. Likewise, there are reports of PKA-independent effects of cAMP-elevating agents in respect of repression of cytokine release and apoptotic responses [23, 24]. Therefore, it is highly likely that further nonclassical, yet biologically significant, mechanisms of $\beta_2$-adrenoceptor signalling remain to be elucidated [19, 21, 25–28].

## ADRENOCEPTORS IN THE LUNG

Adrenoceptors are members of a large family of related G-protein-coupled receptors (GPCRs) and are activated by the endogenous hormones adrenalin and noradrenalin. There are two main groups of adrenoceptor and these have been classified as α- and β-subtypes, which are encoded by at least nine distinct genes ($\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1D}$, $\alpha_{2A/D}$, $\alpha_{2B}$,$\alpha_{2C}$, $\beta_1$, $\beta_2$ and $\beta_3$) [29]. $\alpha_1$-Adrenoceptors can couple *via* the G-protein, Gq, to phospholipase C (PLC), ultimately leading to an increase in the cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) [30]. Accordingly, $\alpha_1$-adrenoceptor agonists promote $Ca^{2+}$-dependent responses, typically smooth muscle contraction. Conversely, agonism of $\alpha_2$-adrenoceptors can lead to an inhibitory G-protein (Gi)-mediated inhibition of AC that may act to prevent smooth muscle relaxation [30]. Despite evidence for α-adrenoceptors in the lung, neither receptor subtype has a clear role in regulating human ASM tone [31]. This is in contrast to all β-adrenoceptor subtypes, which can activate AC through Gs and could, in theory, promote smooth muscle relaxation [30].

It is noteworthy that the $\beta_3$-adrenoceptor can also couple to Gi and, therefore, inhibit AC. This finding may have significance for epithelial cell function, where functional $\beta_3$-adrenoceptor has been described, but not for ASM, which seemingly lacks expression of this subtype [32–34]. In addition to being widely expressed on ASM [31], $\beta_2$-adrenoceptors are also expressed on

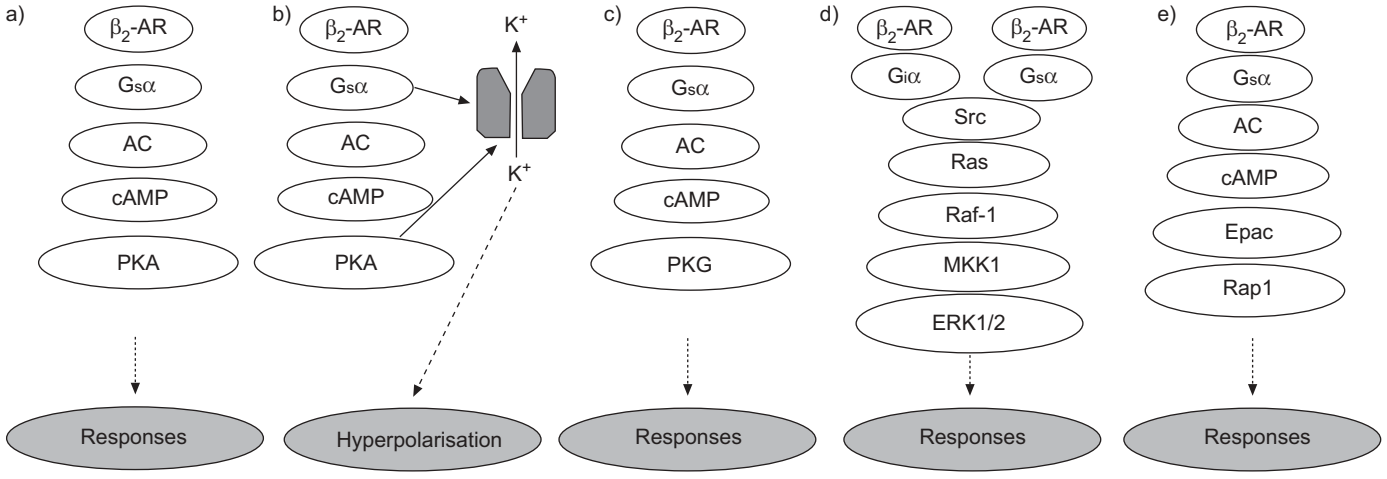


**FIGURE 1.** Multiple pathways of cyclic adenosine monophosphate (cAMP)-dependent signalling. a) Agonism of $\beta_2$-adrenoceptors ($\beta_2$-AR) in the membrane of human airways smooth muscle (ASM) and other cells results in the liberation of stimulatory G-protein (Gs) subunit α from the αβγ heterotrimeric guanine nucleotide-binding protein. The free α subunit then augments the basal activity of one or more isoforms of adenylyl cyclase (AC), resulting in an increase in the formation of cAMP from adenosine triphosphate (ATP). cAMP binds to the regulatory subunits of protein kinase (PK) A and, thereby, promotes the release of the catalytic subunits, which phosphorylate target proteins to bring about changes in cell function. b) Targets of PKA include potassium channels (*e.g.* large-conductance calcium-activated potassium channel and ATP-sensitive potassium channel) that open upon phosphorylation, resulting in the efflux of $K^+$ from the cell down its concentration gradient leading to membrane rectification (repolarisation). In ASM, $K^+$ efflux reduces the excitability of the cell and facilitates ASM relaxation. Gating of $K^+$ channels may also be effected by the direct interaction of Gsα with the channel independently of cAMP and PKA. c) $\beta_2$-AR agonists can also promote the cAMP-mediated activation of PKG (so-called cross-activation), leading to functional responses in the airways such as smooth muscle relaxation. d, e) More novel $\beta_2$-AR signalling cascades include the activation of the tyrosine kinase, Src, *via* either inhibitory G-protein (Gi) subunit α or through Gsα leading to Ras, Raf-1, mitogen-activated protein kinase kinase (MKK) 1 and extracellular signal-regulated kinase (ERK) activation (d) and the binding and activation by cAMP of exchange proteins directly activated by cAMP (Epac) independently of PKA, leading to Rap-1-dependent responses (e).

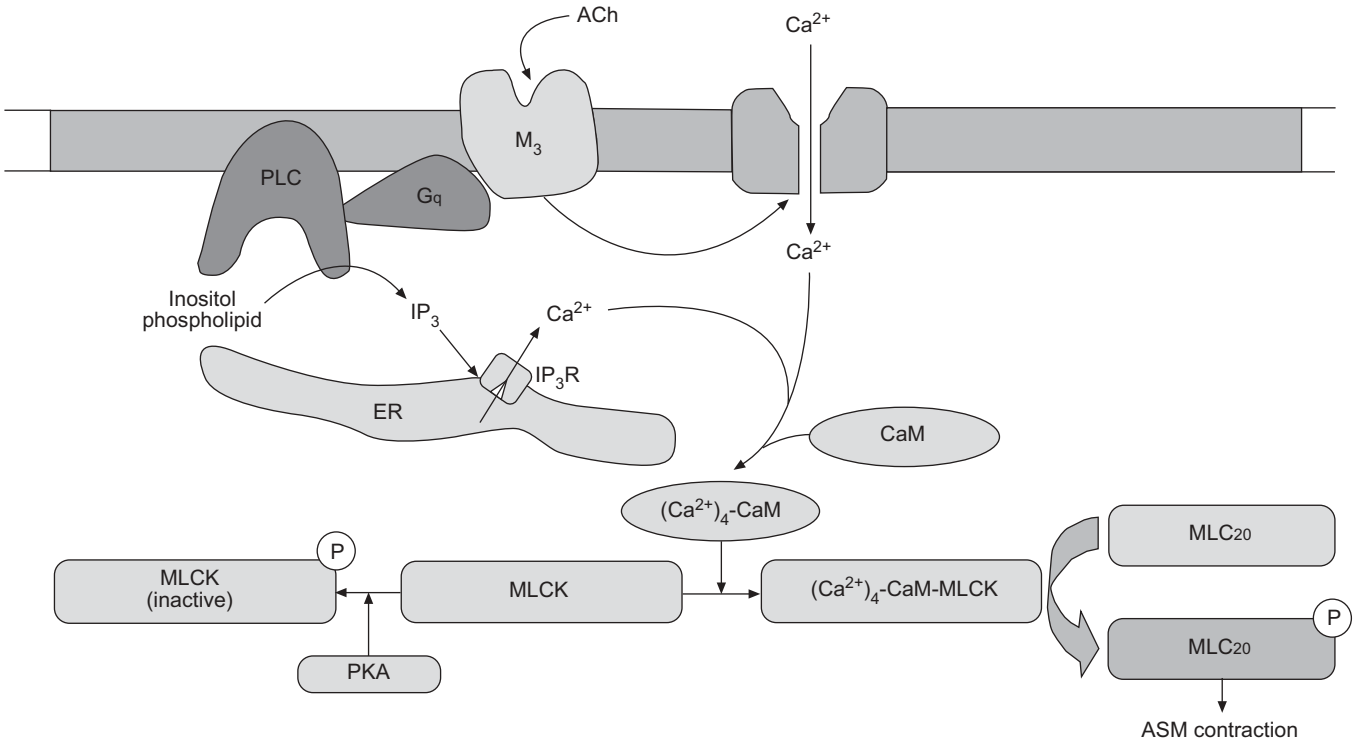


**FIGURE 2.** Contraction of airways smooth muscle. Contractile agonists, such as acetylcholine (ACh), bind to G-protein ($G_q$)-coupled muscarinic $M_3$ receptors on the plasma membrane, resulting in the activation of phospholipase C (PLC) and the subsequent production of inositol 1,4,5-trisphosphate ($IP_3$) as a product of inositol phospholipid hydrolysis. $IP_3$ binds to ligand-gated $IP_3$ receptors ($IP_3Rs$) on the endoplasmic reticulum (ER) to release $Ca^{2+}$ into the cytoplasm. In addition, many agonists may also promote, either directly or indirectly, the entry of $Ca^{2+}$ from the exterior of the cell *via* the action of $Ca^{2+}$ channels in the plasma membrane. The rise in intracellular $Ca^{2+}$ causes $Ca^{2+}$ to bind to calmodulin (CaM; and subsequently myosin light chain kinase (MLCK)) with the formation of a catalytically active $(Ca^{2+})_4$-CaM-MLCK complex, which then phosphorylates (P) serine 19 of the 20-kDa regulatory light chain of myosin (MLC20) to promote actin–myosin cross-linking and smooth muscle contraction. PKA: protein kinase A.

many pro-inflammatory and immune cells, including mast cells [35], macrophages [36], neutrophils [37], lymphocytes [38], eosinophils [39], epithelial and endothelial cells [40, 41], and both type I and type II alveolar cells [10, 42, 43]. Thus, many cell types within the lung that are implicated in the pathogenesis of asthma are potential targets of inhaled $\beta_2$-adrenoceptor agonists.

## $\beta_2$-ADRENOCEPTOR-MEDIATED RELAXATION

It is the profound bronchodilator response effected by $\beta_2$-adrenoceptor agonists that is most critical to asthma therapeutics. Several, possibly complementary, mechanisms have been advanced to explain how agonism of $\beta_2$-adrenoceptors on ASM cells promotes relaxation (see below), although none are entirely satisfactory. This lack of a unifying concept strongly suggests that understanding of this important process is fundamentally incomplete and prompts speculation that additional components of $\beta_2$-adrenoceptor signalling, intimately involved in regulating ASM tone, remain to be defined.

### *Reduced sensitivity of the contractile proteins to calcium*

Generically, agonist-dependent contraction of ASM requires an increase in $[Ca^{2+}]_c$, which can originate extracellularly (entering through $Ca^{2+}$ channels in the plasma membrane) or from intracellular stores (fig. 2). This effect results in the $Ca^{2+}$/calmodulin(CaM)-dependent activation of myosin light chain (MLC) kinase (MLCK), phosphorylation of the 20-kDa MLC (MLC20) at serine (Ser)[19] and subsequent muscle contraction (fig. 2). Conversely, many texts cite the phosphorylation of MLCK by PKA as being an event intimately related to $\beta_2$-adrenoceptor-mediated relaxation (fig. 2) [44]. Indeed, this phosphorylation event reduces the ability of MLCK to phosphorylate MLC20 at Ser[19] by increasing its requirement for $Ca^{2+}$/CaM (*i.e.* it reduces its sensitivity to $Ca^{2+}$; fig. 3) [45–47]. This mechanism also adequately explains the finding that both forskolin and isoprenaline reduce force generation in intact depolarised tracheal smooth muscle at a constant $[Ca^{2+}]_c$; similar data have also been obtained with the addition of PKA to Triton X-100-permeabilised tracheal smooth muscle [48–50]. The additional finding that contraction of tracheal smooth muscle by the $M_3$ muscarinic receptor agonist, carbachol, correlates with MLC20 phosphorylation and that this effect is reduced by isoprenaline also supports the above hypothesis [51]. However, at longer time points, $[Ca^{2+}]_c$ and MLC20 phosphorylation decline towards resting levels, whereas smooth muscle force is maintained. These data indicate that $\beta_2$-adrenoceptor-mediated relaxation of ASM is a complex process involving mechanisms other than, or in addition to, phosphorylation of MLCK [51, 52]. Several studies have provided data that are completely inconsistent with the concept that PKA-mediated phosphorylation of MLCK reduces smooth muscle force. For example, it has been reported that, in bovine tracheal smooth muscle, $\beta_2$-adrenoceptor agonists have no effect on the proportion of MLCK in the activated state at

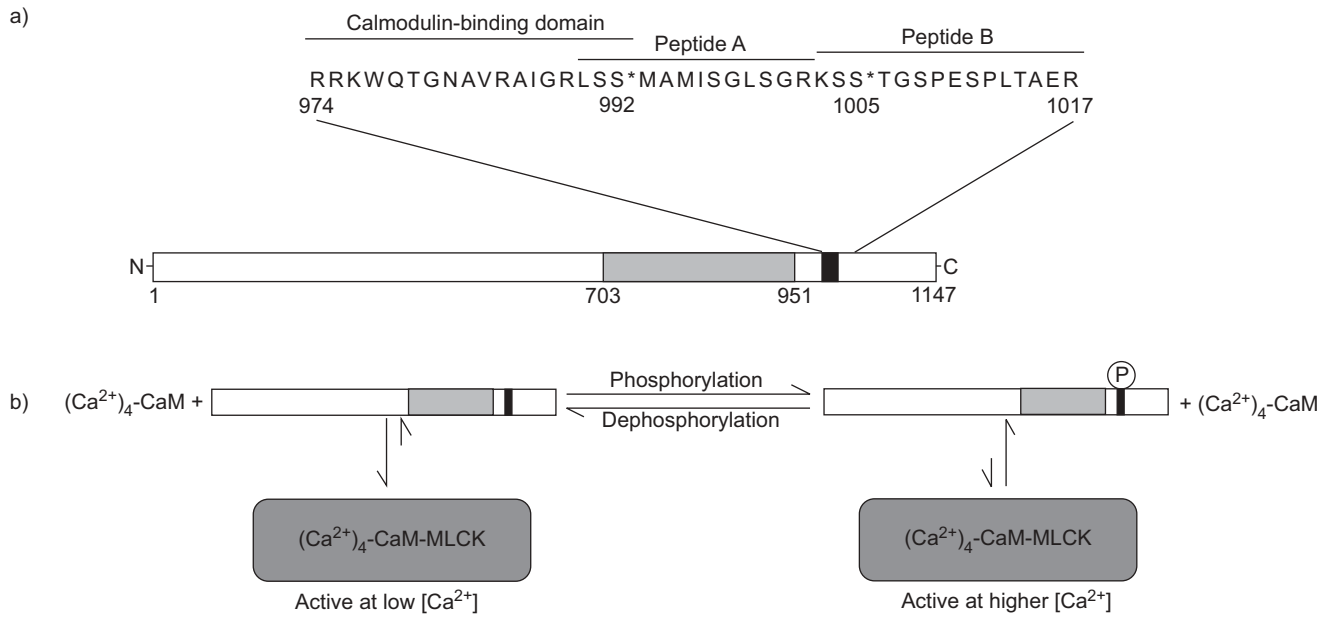


**FIGURE 3.** Activation of myosin light chain kinase (MLCK) by calcium-calmodulin (CaM). a) Schematic representation of the 1,147-amino-acid rabbit smooth muscle MLCK. The catalytic core (amino acids 703–951; ■) and CaM-binding domain (■) are shown, along with an expanded sequence showing part of the CaM-binding domain and its proximity to the tryptic peptides A and B, which are phosphorylated by protein kinase (PK)A. The putative PKA phosphorylation sites (*: serine (Ser) residues 992 and 1005). b) Phosphorylation (P) of a Ser residue (probably $Ser^{992}$) within the peptide A region reduces the ability of $(Ca^{2+})_4$-CaM to bind and activate MLCK. Thus, MLCK phosphorylated in this region requires a higher $[Ca^{2+}]$, at a fixed CaM concentration, to achieve enzyme activation. Unphosphorylated enzyme is more readily activated by $Ca^{2+}$ at a fixed CaM concentration. R: arginine; K: lysine; W: tryptophan; Q: glutamine; T: threonine; G: glycine; N: asparagine; A: alanine; V: valine; I: isoleucine; L: leucine; S: serine; M: methionine; P: proline; E: glutamic acid. (Modified from [11].)

concentrations that caused relaxation [52]. Furthermore, *in vitro*, PKA phosphorylated MLCK at two distinct sites, which are defined by separate tryptic fragments corresponding to amino acids 990–1002 and 1003–1017 in rabbit smooth muscle MLCK (fig. 3) [11, 46, 53, 54]. These fragments have been designated A and B, respectively, and it appears that only phosphorylation within the A fragment, which maps next to the CaM-binding domain, is responsible for the inactivation of MLCK, by reducing $Ca^{2+}$/CaM binding (fig. 3) [11, 46, 53, 54]. Importantly, only minimal change in the phosphorylation within the A site, or indeed MLCK, occurs following exposure of the tissue to cAMP-elevating agents under conditions in which force was shown to be greatly diminished. Thus, there is an absence of a causal relationship between MLCK and muscle relaxation [52, 53]. Therefore, the role of MLCK phosphorylation is equivocal and may not represent the primary mechanism of PKA-mediated $Ca^{2+}$ desensitisation [53]. It is worth noting that the peptides, A and B, contain a number of other potential phospho-acceptor sites. Indeed, the MLCK A site has been shown to be a substrate for CaM-dependent protein kinase II (CaMKII; fig. 4) [55, 56]. As CaMKII is highly $Ca^{2+}$ sensitive, the regulation of MLCK activity *via* this kinase may normally represent a mechanism of classical negative feedback control rather than a primary site for PKA-mediated relaxation (fig. 4) [54]. Finally, MLCK can also become phosphorylated on other residues in response to β-adrenoceptor agonists, raising the possibility of alternative unexplored regulatory mechanisms [53].

The phosphorylation status of $MLC_{20}$ at $Ser^{19}$ [54] is also controlled by myosin-bound protein phosphatase 1 (PP-1M) [57], which opposes the activity of MLCK. PP-1M, often referred to as MLC phosphatase (MLCP) [58], or smooth muscle myosin phosphatase [59], consists of a catalytic subunit of 38 kDa, which is identical to protein phosphatase 1c (PP1c), and two other proteins of ~20 kDa ($M_{20}$) and ~130 kDa (fig. 4) [59, 60]. The function of the smaller subunit is currently unclear, whereas the larger subunit, myosin phosphatase target subunit (MYPT1), or myosin-binding subunit, is responsible for providing substrate specificity by virtue of binding both PP1c and myosin [59]. As stated above, it is the relative activities of MLCK and MLCP that regulate the sensitivity of the contractile apparatus to $Ca^{2+}$. Although it is well established that MLCK activity is controlled by $Ca^{2+}$/calmodulin, it is now apparent that MLCP activity may also be regulated to a high degree, and this represents a primary mechanism involved in the regulation of smooth muscle contractility [58, 59]. Thus contractile agonists lead to the phosphorylation of MYPT1 and another protein, PKC-activated protein phosphatase inhibitor 17 (CPI-17), which appears to be primarily responsible for inactivating MLCP in smooth muscle, with a consequent enhancement of force development [61–63]. Interestingly, the phosphorylation of CPI-17 at threonine ($Thr^{38}$) [64, 65] and MYPT1 at $Thr^{696}$ can occur *via* a range of kinases, including PKC [64], integrin-linked kinase [66], p21-activated kinase [67] and Rho-associated protein kinase (ROCK). Therefore, MLCP and hence $MLC_{20}$ phosphorylation are potentially under the control of multiple kinase signalling cascades (fig. 3) [58, 68–70]. In addition, it has been reported that phosphorylation of MYPT1 by ROCK at other sites ($Thr^{853}$ in humans) promotes its dissociation from myosin and thereby reduces the activity of

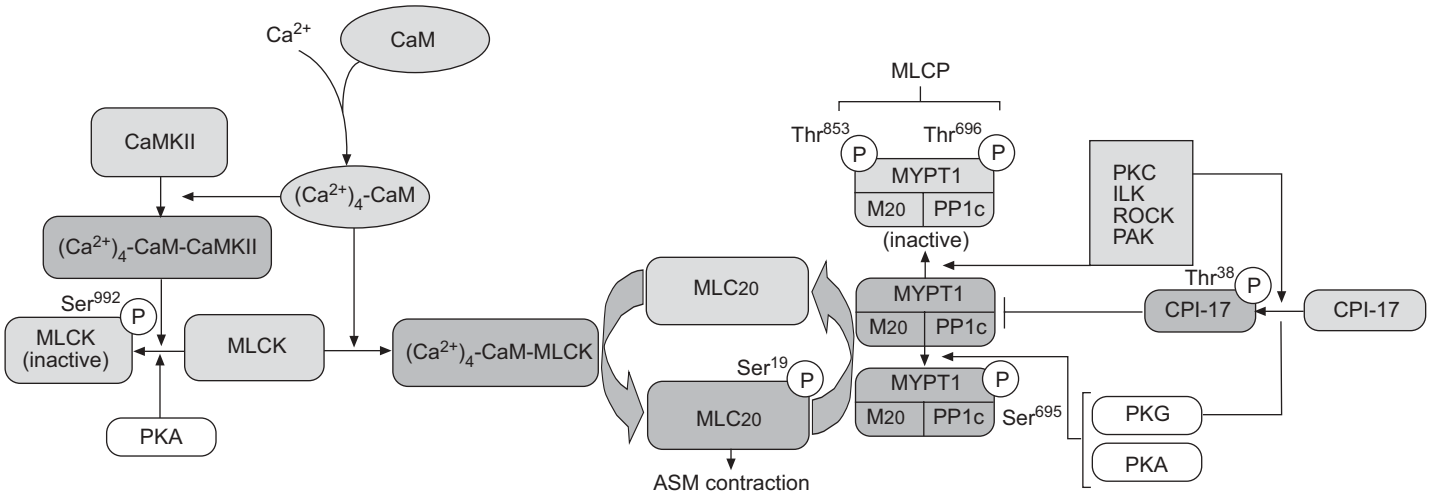


**FIGURE 4.** Opposing activities of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) in the control of airways smooth muscle contraction. The rise in intracellular $Ca^{2+}$ concentration in response to contractile agonist (fig. 3) results in the formation of $(Ca^{2+})_4$-calmodulin (CaM) complexes, which, in turn, activate MLCK. Active MLCK phosphorylates (P) serine $(Ser)^{19}$ of the 20-kDa regulatory light chain of myosin ($MLC_{20}$) to promote smooth muscle contraction. However, $(Ca^{2+})_4$-CaM can also activate CaM-dependent protein kinase II (CaMKII), which phosphorylates MLCK in the A site ($Ser^{992}$) in order to prevent activation by CaM. This is the previously described site for phosphorylation by protein kinase (PK)A. $Ser^{19}$ phosphorylation of $MLC_{20}$ is also regulated by the activity of MLCP. MLCP is a multi-protein complex consisting of protein phosphatase (PP)1c, a 20-kDa subunit ($M_{20}$), the function of which is unknown, and a targeting subunit known as myosin phosphatase target subunit (MYPT1). The primary activity of MLCP is regulated by phosphorylation of MYPT1 by a number of pro-contractile kinases, resulting in a reduced affinity of the phosphatase for myosin and leading to the inactivation of MLCP. In addition, the activity of MLCP towards myosin is also repressed by PKC-activated PP inhibitor 17 (CPI-17), which is itself activated by phosphorylation at threonine (Thr) 38 by pro-contractile kinases. Finally, both PKG and PKA may maintain MLCP activity by targeting MYPT1, at $Ser^{695}$, thereby preventing the adjacent $Thr^{696}$ phosphorylation, which is responsible for switching off MLCP activity. In addition, PKG appears to prevent CPI-17 phosphorylation and activation *via* as yet uncharacterised mechanisms. ILK: integrin-linked kinase; ROCK: Rho-associated protein kinase; PAK: p21-activated kinase. —|: inhibition.

MLCP (fig. 4) [70]. With respect to ASM relaxation, there are data to suggest that β-adrenoceptor agonists may activate MLCP and that this may play an important role in reducing tone [71]. Moreover, MLCP can also be activated by the cyclic guanosine monophosphate (cGMP)/cGMP-dependent PKG pathway. Thus, relaxation of arterial smooth muscle by the nitric oxide donor, sodium nitroprusside, is associated with reduced $MLC_{20}$ phosphorylation [72]. These events correlate with elevated levels of cGMP, increased MLCP activity and a concomitant loss of CPI-17 phosphorylation [72]; the cGMP analogue, 8-bromo-cGMP, is also reported to reduce CPI-17 phosphorylation (fig. 4) [73]. Taken together, these data are potentially significant as cAMP can cross-activate PKG in smooth muscle (fig. 1c) [74], and studies using cyclic nucleotide analogues support a role for PKG [75] rather than PKA in reducing tracheal smooth muscle tone (see below). A further mechanism that would prevent agonist-induced downregulation of MLCP was also recently proposed for PKG and PKA [76]. Thus phosphorylation of the MLCP targeting subunit, MYPT1, at $Ser^{695}$, was not found to affect MLCP phosphatase activity. Instead, $Ser^{695}$ phosphorylation blocked the subsequent phosphorylation at a nearby site ($Thr^{696}$) that is involved in phosphatase inactivation by other kinases (fig. 4). Thus cyclic nucleotide-dependent phosphorylation of MYPT1, at $Ser^{695}$, prevents the ability of pro-contractile kinases to inactivate the phosphatase *via* phosphorylation at $Thr^{696}$. In this manner, cyclic nucleotides may maintain MLCP activity to promote smooth muscle relaxation.

More recent studies have suggested that mitogen-activated protein (MAP) kinases may also impact on contractile responses in airways [64, 77–79] and other types of smooth muscle [80, 81]. In particular, $MLC_{20}$ are the proposed substrate of extracellular signal-regulated kinase (ERK)1 and 2 [82–84]. In the context of $\beta_2$-adrenoceptor-mediated relaxation, MAP kinase phosphatase-1 (MKP-1), which dephosphorylates and inactivates both ERK and p38 MAP kinase, is transcriptionally activated by cAMP-elevating agents, including the β-adrenoceptor agonist, isoprenaline, by a PKA-dependent mechanism [85, 86]. Therefore, the induction of MKP-1 may, as has been recently described for glucocorticoids [87], represent a novel mechanism by which $\beta_2$-adrenoceptor agonists, upon repeated use, can suppress smooth muscle contraction.

### *Calcium-activated potassium channels and membrane hyperpolarisation*

Another biologically significant effect of $\beta_2$-adrenoceptor agonists is membrane hyperpolarisation [11]. In ASM, this response is mediated through the activation of $K^+$ channels in the plasma membrane, which counteracts the electrical excitation and subsequent $Ca^{2+}$ influx that contribute $Ca^{2+}$ to contraction [88]. Of the four main classes of $K^+$ channel, it is the $Ca^{2+}$-activated $K^+$ channels that appear to be the most critical [88, 89], in particular the large- or big-conductance channel ($BK_{Ca}$) [90]. These channels, which are composed of four subunits, are abundant in ASM and are a substrate for PKA [89, 91–93]. Furthermore, their role in regulating ASM contractility is suggested from the finding that pharmacological blockade of these channels with charybdotoxin, and the more selective iberiotoxin, prevents hyperpolarisation and $\beta_2$-adrenoceptor-mediated relaxation [93–96].

More recent studies on the role of $BK_{Ca}$ in $\beta_2$-adrenoceptor-mediated relaxation point to the existence of both cAMP-dependent and -independent mechanisms, as well as a role for the cGMP/PKG signalling cascade [97, 98]. Indeed, there is evidence for direct coupling of $BK_{Ca}$ to Gs, which provides an explanation for the cholera toxin- and $\beta_2$-adrenoceptor-mediated relaxation of guinea pig trachea that prevails in the presence of AC inhibitors (fig. 1b) [21, 22, 97]. Similarly, in vascular smooth muscle, isoprenaline is reported to modulate $BK_{Ca}$ activity in a membrane-delimited manner by a mechanism involving Gi [99], suggesting promiscuous coupling of the $\beta_2$-adrenoceptor. Interestingly, in that tissue, the degree to which $BK_{Ca}$-blocking drugs were effective at preventing $\beta_2$-adrenoceptor-mediated relaxation was highly dependent upon the concentration of agonist [99].

More direct evidence for the coupling of $\beta_2$-adrenoceptor signalling to $BK_{Ca}$ is the finding that co-expression of these proteins in Xenopus oocytes resulted, in the presence of agonist, in channel activation that was abolished either by inhibitors of PKA or following mutation of the consensus PKA phosphorylation sites within the $BK_{Ca}$ $\alpha$ subunit (fig. 1b) [100]. More recent experiments have identified a multi-protein signalling complex that is formed from a direct association of the $BK_{Ca}$ $\alpha$ subunit, $\beta_2$-adrenoceptor and AKAP79/150 (now classified as AKAP5) through a mechanism that may involve leucine-zipper protein–protein interactions [101, 102]. This unique signalling complex may also include an L-type voltage-gated $Ca^{2+}$ channel ($Ca_v1.2$), resulting in a highly localised signalosome that mediates $Ca^{2+}$- and phosphorylation-dependent modulation of $BK_{Ca}$ currents [101].

Notwithstanding the fact that some investigators contend the importance of $BK_{Ca}$ in $\beta_2$-adrenoceptor-mediated relaxation of ASM [88], a potentially significant level of complexity is raised by the knowledge that the pore-forming $\alpha$ subunit of $BK_{Ca}$, encoded by the *slowpoke* gene (*slo1*), exists as multiple splice-variants. One of these $\alpha$ splices (the ZERO isoform) is activated following phosphorylation by PKA, whereas another variant expressing a 59-amino-acid cysteine-rich exon at splice site 2, called the STREX-1 isoform, is inhibited [103]. Moreover, in ASM, these variants of the $BK_{Ca}$ $\alpha$ subunit show differential sensitivity to PKA and PKG [104]. Interestingly, activation of $BK_{Ca}$ $\alpha$ subunit activity by PKA requires phosphorylation of all four members of the $\alpha$ tetramer, whereas inactivation appears to require only a single PKA-mediated phosphorylation of the respective isoform [105]. Even more fascinating is the finding that the glucocorticoid, dexamethasone, is able to block the PKA-dependent inhibition of the STREX-1 isoform, but is without effect on the PKA-mediated activation of standard (ZERO) $\alpha$ subunits [106]. These extraordinary data provide a mechanism by which glucocorticoids may selectively enhance $BK_{Ca}$ channel activity and provides yet another insight into the rationale behind the possible enhanced therapeutic benefit of combining a glucocorticoid and a $\beta_2$-adrenoceptor agonist in the treatment of asthma [6].

### *Role of other potassium channels*

Of the other three main groups of $K^+$ channel, it is only the adenosine triphosphate (ATP)-dependent variants ($K_{ATP}$) [107–109] that are currently believed to be functionally important in ASM [90, 110, 111]. However, although $K_{ATP}$ channel openers oppose bronchoconstriction, attenuate airways hyperreactivity and may play a role in $\beta_2$-adrenocepotor-mediated relaxation of vascular smooth muscle, the effect of selective agonists, *per se*, on these channels in human ASM is not well studied [112–114]. Nevertheless, $K_{ATP}$ channels are phosphorylated and activated by PKA in response to cAMP analogues, forskolin and isoprenaline [115, 116], and could, therefore, play a role in $\beta_2$-adrenoceptor-mediated relaxation of ASM (fig. 1b). This possibility needs to be revisited.

### *Modulation of cytosolic free calcium concentration*

In addition to the well-described mechanisms for reducing the $Ca^{2+}$ sensitivity of the contractile apparatus, $\beta_2$-adrenoceptor agonists are generally believed to reduce $Ca^{2+}$ influx into ASM cells [14, 117–120]. Consistent with this effect, a number of studies have found that the inositol 1,4,5-trisphosphate ($IP_3$) receptor ($IP_3R$) is phosphorylated by PKA, apparently resulting in reduced $Ca^{2+}$ release from the endoplasmic reticulum (ER) in response to $IP_3$ [121, 122]. It is now known that there are at least three types of $IP_3R$ and that, in ASM, cAMP and cGMP both promote the PKG-dependent phosphorylation at $Ser^{1755}$ of the type 1 $IP_3R$ that predominates in this tissue [123]. However, the significance of these findings is questionable as it was subsequently found that phosphorylation of $Ser^{696}$ of another protein called $IP_3R$-associated cGMP-dependent protein kinase substrate, which is intimately associated with the $IP_3R$, also inhibited $IP_3$-induced $Ca^{2+}$ release from the ER and that this effect was lost if $Ser^{696}$ was mutated to alanine.

Of particular interest is the recent finding that isoprenaline, in single isolated ASM cells, increases the $Ca^{2+}$ content in the peripheral cytosol of the cell by a mechanism that is dependent upon external $Ca^{2+}$ concentration and blocked by ryanodine [124]. In contrast, in the same cell, isoprenaline reduces the concentration of $Ca^{2+}$ in the inner cytosol [124]. Thus, $\beta_2$-adrenoceptor agonists can produce discrete spatially distinct changes in the $Ca^{2+}$ concentration within the cytosol of individual cells that presumably have profound functional implications, including effects on tone.

Another mechanism leading to the release of $Ca^{2+}$ from the ER is *via* one or more ryanodine receptor (RyR), of which there are at least three variants [125, 126]. RyR are intracellular $Ca^{2+}$ release channels and are not usually associated with non-excitable cells [125, 126]. However, a recent study by DU *et al.* [127] identified both RyR1 and RyR3 in murine ASM. Moreover, $Ca^{2+}$ release through these channels mediated agonist (carbachol)-induced tension development, a finding that was consistent with a previous report by HAY *et al.* [128]. Taken together, these data suggest that RyR may represent novel targets for suppressing bronchoconstriction, although it should be noted that the effect of activation of the cAMP/PKA cascade on RyR function appears to be variable [126]. Thus, studies specifically designed to examine the role of RyR in the regulation of ASM tone are clearly warranted.

## LONG-TERM USE OF $\beta_2$-ADRENOCEPTOR AGONISTS

### *Asthma deaths, adverse events and effect of glucocorticoids*

Long-term use of $\beta_2$-adrenoceptor agonists is common in the management of asthma and airways hyperresponsiveness (AHR), which is defined as an increase in the sensitivity of

the ASM to constrictor stimuli [129–132]. Similarly, the ability of short- and long-acting $\beta_2$-adrenoceptor agonists to protect the airways against bronchoconstrictor stimuli and to promote bronchodilatation may be partially lost with time following long-term use [2, 129, 131, 133–140]. Indeed, there is a greater tendency for bronchodilator tolerance to develop to long-acting $\beta_2$-adrenoceptor agonists compared to their short-acting counterparts, which may relate to some aspect of their prolonged duration of agonism [141, 142]. The alteration in ASM sensitivity to spasmogens is believed to predispose asthmatic subjects who use $\beta_2$-adrenoceptor agonist on a regular basis to episodes of acute bronchoconstriction or ''a loss of asthma control'' [143]. Thus, long-term $\beta_2$-adrenoceptor agonist use is associated with an increased incidence of asthma exacerbations and other markers of morbidity and mortality [143–145]. Indeed, this finding is consistent with the many studies that have found that regular use of $\beta_2$-adrenoceptor agonists, in particular full agonists *e.g.* fenoterol, is associated with elevated bronchial responsiveness and asthma-associated deaths [7, 146]. Accordingly, the current recommendation of most national asthma guidelines is to use short-acting $\beta_2$-adrenoceptor agonists as relievers (*i.e.* on an as-needed basis) and not as a regular prophylactic therapy. More recently, the issue of $\beta_2$-adrenoceptor agonist safety has arisen again in respect of long-acting compounds [141]. Thus, studies published in 1993 indicated that, although patients taking salmeterol generally showed better control of their asthma, a small but nonsignificant increase in the number of deaths in the salmeterol treatment group was noted [147]. That result led to the Salmeterol Multi-centre Asthma Research Trial (SMART), which also revealed a slightly increased risk of death in subjects of certain genetic backgrounds [148]. The issue of genetic susceptibility was again highlighted by findings indicating that certain $\beta_2$-adrenoceptor polymorphisms may affect patient phenotype and outcome in response to $\beta_2$-adrenoceptor agonist treatment [149], implying that genetic analysis may, in the future, be diagnostically useful in tailoring individual treatment regimens. The results of the SMART further prompted a warning by the US Food and Drug Administration, and the current recommendation from most key sources is that long-acting $\beta_2$-adrenoceptor agonists should only be used in conjunction with inhaled glucocorticoids [150–153]. Although the balance of expert opinion is that the benefits of long-acting agonists, such as salmeterol, when taken alongside glucocorticoids, far outweigh the potential risk, there is still a pressing need to provide a rational molecular basis for these adverse effects. This will be especially important given the probable introduction before 2010 of ultra-long-acting $\beta_2$-adrenoceptor agonists such as carmoterol, arfomoterol (*R,R*-formoterol), indacaterol and GSK 159797 (GlaxoSmithKline, Stevenage, UK) [142].

### *Mechanistic basis for adverse effects*

#### Receptor desensitisation

One widely touted explanation for the reduction in both the bronchodilator and bronchoprotective actions of these drugs is that regular treatment leads to $\beta_2$-adrenoceptor desensitisation (*i.e.* a state of refractoriness that ensues following prolonged exposure or repeated application of an agonist [129, 141]). Receptor desensitisation (as discussed below) can occur *via* a variety of processes, including uncoupling of the receptor from $G_s$, receptor internalisation [3, 10, 13], upregulation of cAMP PDE [154–157] and downregulation of $G_s$ [158]. However, receptor desensitisation may not fully account for all of the effects of prolonged high-level dosing with $\beta_2$-adrenoceptor agonists [9]. One unexpected observation, which may be of clinical relevance, is the finding that agonism of the $\beta_2$-adrenoceptor can lead to overexpression of PLC$\beta_1$ in ASM [159]. Using $\beta_2$-adrenoceptor knockout mice, MCGRAW *et al.* [159] made the novel observation that methacholine (MCh)- and 5-hydroxytryptamine (5-HT)-induced bronchoconstriction were markedly reduced compared with the same response evoked in wild-type animals. Conversely, MCh- and 5-HT-induced bronchoconstriction in transgenic animals overexpressing two-fold cell surface $\beta_2$-adrenoceptors was significantly enhanced. Thus, contrary to expectation, agonism of $\beta_2$-adrenoceptors in mice augments signalling through GPCRs utilised by contractile agonists. Subsequent studies confirmed that inositol phosphate accumulation evoked by MCh, 5-HT and U-46619 (a thromboxane mimetic) was suppressed and augmented in knockout and transgenic overexpressing mice, respectively [159]. These data led to the idea that $\beta_2$-adrenoceptors antithetically regulate the actions of $Ca^{2+}$-mobilising contractile agonists and can activate mechanisms that simultaneously promote bronchoconstriction and bronchodilatation. Further investigations have implicated PLC$\beta_1$ as the point of regulation since expression of this enzyme was enhanced and inhibited, respectively, in $\beta_2$-adrenoceptor transgenic and knockout mice [159]. Taken together, these results provide an unanticipated and intriguing explanation for the clinical finding that long-term treatment of asthmatic subjects with $\beta_2$-adrenoceptor agonists can promote AHR and increase the incidence of exacerbations. The mechanism(s) by which $\beta_2$-adrenoceptor agonists upregulate PLC$\beta_1$ are unknown. However, classical pharmacology would predict that this effect is related to the intrinsic efficacy of the agonist and that partial agonists might be less prone to antithetically regulating signalling through PLC$\beta_1$. Despite the elegance of these studies, it should be borne in mind that much of these data were obtained in mice following manipulation of cell surface $\beta_2$-adrenoceptor number, and it is currently unclear to what extent such processes occur in human ASM in an *in vivo* setting.

#### Racemic formulations

All $\beta_2$-adrenoceptor agonists used clinically are racemates, in which the *R*-enantiomer is generally the more active component relative to the *S*-enantiomer [160]. The ability now to produce highly pure stereoisomers has led to the view that the less active or inactive *S*-enantiomer may be responsible for evoking some of the unwanted paradoxical responses by an unknown mechanism(s) that is unrelated to $\beta_2$-adrenoceptor desensitisation (see above). Moreover, pharmacokinetic studies have found that the metabolic clearance of *S*-salbutamol occurs at a significantly lower rate than that of *R*-salbutamol, such that the unwanted effects may persist after the beneficial actions have worn off [161]. Evidence is available that *S,S*-formoterol (inactive) may also accumulate *in vivo* relative to the active *R,R*-stereoisomer due to differential rates of metabolism [162]. In view of these data, it is perhaps not surprising that many new long-acting $\beta_2$-adrenoceptor agonists in clinical development are pure pharmacologically active enantiomers [142].

### Promiscuous G-protein coupling

Another mechanism worthy of consideration that could contribute to the undesirable actions of long-term $\beta_2$-adrenoceptor agonist treatment is the finding that $\beta_2$-adrenoceptors can couple to multiple effectors *via* distinct G-proteins [9, 163]. Studies conducted in the 1980s and early 1990s established that purified $\beta_2$-adrenoceptor or a peptide corresponding to the third intracellular loop of the protein could activate pure $G_i$ in a reconstituted cell-free system [164, 165], indicating that this promiscuous coupling could also occur in intact cells and, arguably, *in vivo*. It is now firmly appreciated that $\beta_2$-adrenoceptors may couple to multiple guanosine triphosphate (GTP)-binding proteins, including $G_i$ [166, 167], leading to $G\beta\gamma$-dependent activation of the MAP kinase/ERK cascade (fig. 1d). Since this pathway is undoubtedly activated in asthma and chronic obstructive pulmonary disease and may contribute to the inflammation and even remodelling of the airway wall [168], it is tempting to speculate that further activation of ERK-dependent signalling may account for some of the adverse clinical observations made with $\beta_2$-adrenoceptor agonists described above [169, 170]. Such effects, as are often the case, require appropriate validation in primary cells relevant to the airways or suitable animal models.

### Other explanations

Undesirable effects of $\beta_2$-adrenoceptor agonists may also be related to several other actions. For example, chronic treatment of human bronchi with fenoterol enhances contractile responses to endothelin *via* an effect that may be due to the sensitisation of the transient receptor potential vallinoid 1 channel [171, 172]. In addition, repression of eosinophil apoptosis [173, 174], induction of neurokinin (NK)-2 receptor expression [175, 176], and upregulation of histamine $H_1$ receptor expression [177] are all examples of responses that would be considered undesirable in the context of asthma pathogenesis. Interestingly, glucocorticoids tend to reverse many of these effects, demonstrating the highly beneficial potential of combined $\beta_2$-agonist/corticosteroid therapy [6]. Indeed, glucocorticoids enhance $\beta_2$-adrenoceptor expression and function [178–180], resensitise $\beta_2$-adrenoceptors [181], increase $G_s\alpha$ expression [182], enhance eosinophil apoptosis [173] and reverse the upregulation of NK-2 receptor expression [175].

## EVOLVING CONCEPTS OF $\beta_2$-ADRENOCEPTOR SIGNALLING

There are numerous reports in many cells, including ASM and pro-inflammatory cells, documenting the existence of cAMP-dependent, yet PKA-independent, responses. Data accrued since the mid-1990s also provide intriguing evidence that GPCRs may not signal individually but as homo- or even heterodimers or higher-order oligomers. Another major advance has been the unequivocal demonstration of compartmentalisation of cAMP-dependent hormone action. In the following sections, these recent advances are discussed in the context of $\beta_2$-adrenoceptor signalling and asthma therapy together with data obtained from studies employing transgenic mice overexpressing pulmonary $\beta_2$-adrenoceptors.

### *$\beta_2$-Adrenoceptor oligomerisation*

Traditionally, it was thought that GPCRs existed and functioned as discrete monomers, and that the stoichiometry of receptor, G-protein and effector interaction was equivalent (*i.e.* 1:1:1) [183]. However, there are many studies demonstrating cooperativity in agonist binding to GPCRs, which led to the suggestion that each receptor may, indeed, assemble and signal as part of a larger multi-receptor array. Since the early 1990s, the concept of GPCR dimers or higher-order oligomers has gained general acceptance, and persuasive evidence for this phenomenon in transformed cell systems is now available [184]. It should be noted that oligomerisation has not yet been demonstrated in nontransformed cells relevant to airways biology and so the significance of the findings discussed below is currently unclear. Nevertheless, the possibility that GPCRs can function as oligomers seems likely and is worthy of discussion in this article. As most current methods cannot distinguish between dimers and oligomers, the former term is used throughout the rest of this discussion as it represents the minimum oligomeric form of the receptor [185]. Nevertheless, this term is not meant to exclude the possibility of the existence of higher-order GPCR complexes.

Using the technique of saturated bioluminescence resonance energy transfer (BRET), it has been estimated that 82% of human $\beta_2$-adrenoceptors expressed on human embryonic kidney (HEK) 293T cells exist as dimers [186, 187]. Moreover, studies by HEBERT *et al.* [188] demonstrated that a peptide corresponding to transmembrane domain VI of the $\beta_2$-adrenoceptor, which features a putative motif ($Gly^{276}$-$X_3$-$Gly^{280}$-$X_3$-$Leu^{284}$) for intramolecular receptor: receptor interactions at the C-terminus, disrupted dimerisation in a concentration-dependent manner. Significantly, this effect was associated with impaired agonist, but not forskolin- or sodium fluoride-, induced AC activation, indicating that dimeric $\beta_2$-adrenoceptors may be the minimum functional signalling unit.

An important focus of research following the discovery of GPCR dimerisation was the mechanism(s) regulating the formation of receptor complexes. ANGERS *et al.* [189] advanced three possible scenarios: 1) GPCR dimers are stable preformed complexes that are constitutively expressed at the cell surface and are unaffected by ligand binding; 2) GPCR dimers are constitutively expressed at the cell surface but ligands can alter the degree of dimerisation; and 3) ligand binding is a prerequisite for GPCR dimerisation. With respect to the $\beta_2$-adrenoceptor, early studies found a high degree of constitutive dimerisation in *Sf9* cells expressing the recombinant protein, which was increased (albeit modestly) by isoprenaline, an observation that favours scenario two [187, 188]. However, an emerging consensus for family A GPCRs, which include the $\beta_2$-adrenoceptor, is that ligands bind to constitutively expressed dimers and that the apparent increase in ligand-induced dimerisation, implied from BRET studies, probably represents conformational changes of pre-existing dimers rather than the formation of new receptor complexes [187, 190].

Thus the question remains, where and how is $\beta_2$-adrenoceptor dimerisation regulated? It is well established that the assembly within the ER of proteins in general is a common form of quality control used by a cell to permit the export of correctly folded complexes [191]. This process also appears to apply to GPCRs. Indeed, immature forms of the $\beta_2$-adrenoceptor have been recovered as dimers from ER-enriched fractions of HEK 293T cells, suggesting that complex formation takes place early

during receptor biosynthesis [192, 193]. Further support for this hypothesis comes from the fact that mutant $\beta_2$-adrenoceptors that lack the normally expressed heterologous export motif do not leave the ER *en route* to the plasma membrane. Similarly, $\beta_2$-adrenoceptors harbouring the retention signal, which ordinarily is masked if the protein is folded correctly (*i.e.* is functionally competent), still dimerise with wild-type receptors, but, again, do not move to the cell surface. Moreover, disruption of the putative dimerisation motif in transmembrane domain VI (see above) prevents normal trafficking of the receptor to the plasma membrane. Thus, dimerisation is seemingly part of the maturation process of $\beta_2$-adrenoceptors, providing an important mechanism that permits the production of functionally competent ligand recognition and signalling units.

An additional level of complexity is that a GPCR may dimerise with a different, but closely related, receptor. BRET studies have found that $\beta_1$- and $\beta_2$-adrenoceptors can form heterodimers with a binding affinity that is similar to homodimeric $\beta_2$-adrenoceptor complexes [186]. Dimerisation of the $\beta_2$-adrenoceptor with the angiotensin II type 1 receptor [194] and both δ- and κ-opioid receptors has also been described [195]. The latter finding has important consequences for signalling, as each member of the dimer may couple to a different G-protein ($G_s$ and $G_i$, respectively). For example, δ-opioid receptors co-expressed in HEK 293T with $\beta_2$-adrenoceptors undergo rapid isoprenaline-induced endocytosis. Similarly, etorphine (a δ-agonist) promotes the internalisation of $\beta_2$-adrenoceptors in the same system [195]. However, $\beta_2$-adrenoceptors, when co-expressed with κ-receptors, undergo neither opioid- nor isoprenaline-induced endocytosis [195]. Moreover, isoprenaline promotes the phosphorylation of ERK1 and ERK2 in cells expressing heterodimers composed of $\beta_2$-adrenoceptors and δ-receptors, which is essentially lost in cells in which the opioid binding partner is replaced with the κ-receptor [195]. Thus, in expression studies, the composition of GPCR dimers has a marked influence on G-protein coupling, receptor signalling, agonist-induced desensitisation and ligand pharmacology, and could help provide explanations for many of the effects described in the present review [185, 196]. Accordingly, this phenomenon may have significant implications for drug design in the future. Indeed, the crystal structure of the GPCR rhodopsin illustrates that its cytoplasmic surface is too small to accommodate, simultaneously, more than a single point of contact with either the α and βγ subunits of a G-protein [196, 197]. This has consequently led to the suggestion that two receptors might be necessary to satisfy the binding requirements of a single G-protein [197, 198]. It is clear from the preceding discussion that a number of key questions need to be addressed. These include the relevance and prevalence in native tissues of GPCR homo- or heterodimers [199], and whether the biosynthesis of these novel signalling arrays and the functional responses they mediate are affected by a multitude of factors, including airways disease, sexual dimorphism, drug therapy, age or genetic polymorphisms.

### *Studies using transgenic mice overexpressing functional $\beta_2$-adrenoceptors*

Elegant studies by McGRAW *et al.* [200] have revealed new insights into the regulation of ASM tone by $\beta_2$-adrenoceptors. In airway myocytes harvested from the trachea of transgenic mice overexpressing, ~75-fold, $\beta_2$-adrenoceptors, basal AC activity and cAMP content are significantly greater than in smooth muscle cells taken from the trachea of nontransgenic litter mates. In addition, isoprenaline-stimulated cAMP accumulation and AC activity are enhanced further in transgenic animals, indicating that, despite the markedly increased receptor density, maximal constitutive activation of AC was not attained. Controversially, these data imply that the $\beta_2$-adrenoceptor is the rate-limiting component of the receptor/$G_s$/AC signalling cascade, at least in murine airway myocytes and challenges the contention, for which unequivocal empirical data are lacking, that there exists on ASM a large receptor reserve, at maximal response, for $\beta_2$-adrenoceptor agonists used in clinical practice [201–203]. Indeed, if the spare-receptor hypothesis is correct, an increase in receptor density would not be expected to have any major impact on the maximum agonist-induced response.

Functionally, targeted overexpression of $\beta_2$-adrenoceptors on ASM has marked consequences. Thus MCh-induced bronchoconstriction is significantly reduced in transgenic mice in the absence of a $\beta_2$-adrenoceptor agonist compared with animals not expressing the transgene [200]. Furthermore, the magnitude of bronchoconstriction evoked by MCh in nontransgenic animals in the presence of salbutamol is greater than that achieved in transgenic mice not treated with agonist [200]. As identified by the authors, these data are consistent with a multi-state model of GPCRs, in which the effector, AC in this case, is activated by the receptor in the absence of agonist. Obviously, in order to account for this model, it is necessary to propose that the equilibrium in the absence of agonist favours the inactive conformation. In the experiments described by McGRAW *et al.* [200], 75-fold overexpression of ASM $\beta_2$-adrenoceptors allowed sufficient spontaneous coupling to severely limit MCh-induced tone in the absence of agonist. Assuming the pharmacological behaviour of the murine $\beta_2$-adrenoceptor can be extrapolated to humans, these transgenic animals exhibit what may be described as an antiasthma phenotype, which tempts speculation that targeted overexpression of $\beta_2$-adrenoceptors to ASM cells could provide a genetic therapy for asthma [200].

The density of $\beta_2$-adrenoceptors on airway epithelial cells also has an impact on the tone of the underlying smooth muscle. Using the rat Clara cell secretory protein promoter to enhance, two-fold, cell surface $\beta_2$-adrenoceptor number on airway epithelial cells in mice, McGRAW *et al.* [204] found that the dose of MCh required to increase, over baseline, airways resistance by 200% was significantly higher compared with that in nontransgenic litter mates. As in the smooth muscle study described above, the protection afforded against MCh-induced bronchoconstriction in the transgenic animals was the same as that produced by inhaled delivery of salbutamol to mice not expressing the transgene [204]. These data demonstrate that the density of $\beta_2$-adrenoceptors on epithelial cells can regulate ASM tone in the absence of agonist, implying that the degree of spontaneous coupling to AC is increased in transgenic mice. The mechanism underlying this protective effect is unknown, but it is not apparently due to the enhanced release from the epithelium of nitric oxide or prostaglandin $(PG)E_2$ [204]. Thus, the targeted expression of $\beta_2$-adrenoceptors

to the airways epithelium could also provide a novel gene therapy for asthma.

### *Src tyrosine kinases as effectors of $\beta_2$-adrenoceptor agonism*

In addition to activating the classical cAMP/PKA cascade shown in figure 1a, $\beta_2$-adrenoceptor agonists have also been shown to stimulate MAP kinase signalling, although the mechanism underlying this effect is not completely understood. In the original description of this phenomenon, DAAKA *et al.* [169] reported that exposure of HEK 293 cells to isoprenaline led to cAMP/PKA-dependent phosphorylation and desensitisation of the $\beta_2$-adrenoceptor and a consequent switch in receptor coupling from $G_s$ to $G_i$. Activation of MAP kinase then occurred by the sequential activation of a Src→Son of Sevenless (SOS)→Ras→ERK-dependent signalling cascade initiated by $G_i\beta\gamma$ subunits [205–207]. However, in a subsequent study using the same cells, no evidence for $G_s$:$G_i$ switching was found; indeed, FRIEDMAN *et al.* [170] reported that ERK is phosphorylated by a Src-dependent mechanism mediated by the classical $G_s\alpha$→AC→PKA cascade (fig. 1d). Regardless of the precise mechanism of isoprenaline-induced ERK activation, these data clearly implicate Src tyrosine kinases in $\beta_2$-adrenoceptor-mediated signalling. Significantly, this finding is not restricted to HEK 293 cells and seemingly occurs in primary cells of the lung and airways. For example, PP2, a Src tyrosine kinase inhibitor, was recently reported to partially block $\beta_2$-adrenoceptor-mediated actin depolymerisation of ASM cells [208, 209], by a mechanism that was insensitive to pertussis toxin and the MAP kinase kinase (MKK) 1/2 inhibitor, PD098059. The additional observation that cholera toxin mimicked the effect of isoprenaline [209] is consistent with the data of FRIEDMAN *et al.* [170] that a $G_s$/Src-mediated pathway in ASM is responsible for this effect (fig. 1d) [209, 210].

An elevation of cAMP levels also leads to the inhibition of both growth responses and ERK activation in fibroblasts [211–213]. Again, this process appears to involve a PKA-dependent phosphorylation of Src, or a Src-like kinase, leading to the activation of the small GTPase, Rap1, and subsequent repression of the Raf→MKK1/2→ERK cascade [214]. However, it is important to note that, in a different cell line, this signalling cascade can also be activated *via* PKA-dependent phosphorylation of Src [215]. Clearly, these data demonstrate the potential diversity of $\beta_2$-adrenoceptor signalling and highlight the importance of characterising physiologically relevant responses under physiological conditions in nontransformed cells of the tissue of interest.

### *cAMP-guanine nucleotide exchange factors: novel cAMP-dependent effectors*

Until relatively recently, most cAMP-dependent functional responses were generally believed to be mediated by one or more isoforms of PKA. However, in 1998, the world of cAMP signalling underwent radical reshaping, with the discovery of cAMP-activated guanine nucleotide exchange factors (GEFs; cAMP-GEFs), also known as exchange proteins directly activated by cAMP (Epacs) [216–218]. These GEFs function in a manner similar to SOS, which is the GEF responsible for unloading guanosine diphosphate from Ras and promoting Ras-GTP formation and subsequent activation of the Raf→MKK→ERK cascade [218, 219]. Thus, binding of cAMP to cAMP-GEFs allows the activation of an associated small GTPase. In the case of cAMP-GEFI (Epac), Rap1 was suggested to be the downstream G-protein, and has been shown to activate MAP kinase kinase kinase, B-raf and downstream MAP kinase cascades (fig. 1e) [216, 220]. However, the situation in respect of cAMP-GEFs was complicated from the outset by an initial report describing the existence of two cAMP-binding GEFs, cAMP-GEFI and cAMP-GEFII (Epac 2) [217]. In terms of pulmonary physiology, the role of cAMP-GEFs is currently unclear since neither isoform is apparently expressed at a level that is detectable in adult lung (*cf.* foetal lung) [217]. Furthermore, the initial description of a Rap1–B-raf pathway leading to ERK activation may not necessarily occur *via* cAMP-GEFs. Indeed, the use of a cAMP-GEF-selective cAMP analogue that activated Rap1 failed to influence ERK activity, suggesting that effects of cAMP on ERK are independent of Rap1 [221]. In addition, there are a number of other signalling processes and proteins that may also be targeted by cAMP-GEFs, indicating the need to elucidate the functional significance of these novel pathways [218]. Nevertheless, cAMP-GEFs are now being widely examined with a view to explaining the increasing number of observations of cAMP-dependent PKA-independent effects, especially since a number of other putative cAMP-binding proteins do not seem to bind cAMP [222].

### *Activation of PKG*

Another key signalling pathway that needs to be considered more carefully in the context of $\beta_2$-adrenoceptor-mediated relaxation is the cGMP/PKG cascade. Activation of PKG is a well-established mechanism leading to relaxation of smooth muscle [223], including ASM [224–226], which may involve the direct phosphorylation and activation of $BK_{Ca}$ [227]. In addition, PKG can lead to the repression of certain genes that may be of potential significance in airways diseases such as asthma, of which inflammation is a characteristic feature [223, 225, 226]. Of particular interest is the finding that $K^+$-induced contractions of guinea pig tracheal segments were more potently inhibited by cell permeant cGMP analogues than by analogues of cAMP [75]. This inhibitory effect correlated more closely with the ability of the same analogues to activate PKG rather than PKA, suggesting that, in this tissue at least, PKG plays a dominant role in regulating relaxation [75]. The activation of PKG by cAMP [228], which is referred to as cross-activation, has been demonstrated in intact smooth muscle over a concentration range that is remarkably similar to that required to activate PKA, suggesting that it is of physiological relevance (fig.1c) [74, 228]. Further exploration of cross-activation has revealed that the binding of cAMP to PKG may promote autophosphorylation, leading to an increase in the affinity of cAMP for the enzyme [229, 230]. Taken together, these data suggest that PKG is a strong candidate for mediating cAMP-dependent, PKA-independent responses in the airways [25]. This conclusion is supported by the finding that inhibitors of PDE5, a cGMP PDE, may be effective bronchodilators [231] and show independent anti-inflammatory activity [232].

### *Compartmentalisation of $\beta_2$-adrenoceptor signalling*

Agonist-induced acute homologous desensitisation of $\beta_2$-adrenoceptors involves receptor phosphorylation by PKA

and/or one or more GPCR kinases (GRKs), which have the unique ability to recognise the agonist-occupied form of the receptor [233]. In addition, the ability of GRKs to disrupt $\beta_2$-adrenoceptor signalling through phosphorylation is enhanced ~10-fold following the binding of scaffold proteins called β-arrestins [234, 235]. Recently, elegant studies have extended the scaffold functions of β-arrestin to include certain members of the PDE4D family of enzymes, including PDE4D3 and, in particular, PDE4D5 [236, 237]. Thus, following agonism of $\beta_2$-adrenoceptors expressed in HEK 293 cells, a β-arrestin/PDE4D complex forms and is recruited to the receptor, where it limits activation of the cAMP/PKA cascade by simultaneously suppressing AC activity, through receptor desensitisation, and accelerating the removal of cAMP, through enhanced degradation [236]. It appears that the unique amino-terminal region of PDE4D5 confers preferential interaction with β-arrestins and may represent the normal binding partner of this scaffold in intact cells [237]. This targeting of PDE4D to the $\beta_2$-adrenoceptor complex may have highly discrete functional implications for the cell as it will selectively regulate the activity of a pool of PKA that is co-localised to the same subcellular microdomain *via* an interaction with an AKAP [238]. Both AKAP5 [239] and AKAP12 (gravin) [240, 241] have been shown to be recruited to the $\beta_2$-adrenoceptor, although only the latter species is thought to be functionally relevant [238]. Thus, compartmentalisation of signalling allows the level of cAMP to be controlled very tightly within highly discrete intracellular loci, presumably with unique biological consequences, including the extent to which the $\beta_2$-adrenoceptor undergoes PKA-dependent phosphorylation [242].

## NOVEL MECHANISMS OF $\beta_2$-ADRENOCEPTOR DESENSITISATION

A controversial issue that has received considerable attention in the past is whether $\beta_2$-adrenoceptor agonists exhibit anti-inflammatory activity. *In vitro*, it has been known for some time that exposure of purified immune and pro-inflammatory cells, such as eosinophils, mast cells, T-lymphocytes and neutrophils, to $\beta_2$-adrenoceptor agonists generally results in the inhibition of various functional indices of activation [243]. Similarly, acute administration of $\beta_2$-adrenoceptor agonists to humans effectively suppresses inflammatory leukocyte infiltration and stabilises mast cells in response to direct and indirect stimuli [243]. However, there is little evidence that regular administration of these drugs can prevent AHR, the late-phase asthmatic response or the activation *in vivo* of those cells that initiate and perpetuate the chronic inflammation that characterises asthma [2, 244].

The inability of $\beta_2$-adrenoceptor agonists to resolve asthmatic inflammation may be due to the development of tolerance (or desensitisation) and is consistent with the rapid loss of responsiveness of essentially all pro-inflammatory and immunocompetent cells following prolonged exposure to $\beta_2$-adrenoceptor agonists *in vitro* [2, 10]. Two major molecular mechanisms that can account for $\beta_2$-adrenoceptor desensitisation have been extensively described. One of these promotes short-term homologous refractoriness and involves the uncoupling of the receptor from $G_s$ by mechanisms that require phosphorylation of Ser and Thr residues at the C-terminus of the agonist-occupied receptor [245]. This reaction is catalysed by at least three GRK family members (GRK2, GRK3 and GRK5), which are attracted to, and anchored at, the plasma membrane by $G_s\beta\gamma$ heterodimers that are liberated following agonist-induced activation of $G_s$. Signalling through the receptor is then halted by the subsequent binding of β-arrestin, a soluble protein which prevents further coupling to $G_s$ [235]. The $\beta_2$-adrenoceptor is similarly desensitised by PKA following phosphorylation of Ser and Thr residues present within the third intracellular loop of the protein in response to an increase in intracellular cAMP [233, 246]. Evidence is also available that $G_s$ can activate Src tyrosine kinases, which have been shown to bind both β-arrestins and the phosphorylated form of the receptor, as well as activate GRKs (fig. 1d) [205, 207]. Furthermore, the recruitment of kinases (GRKs and PKA) to the receptor complex during the desensitisation process may be specifically targeted, and enhanced, *via* interactions with certain AKAPs [240, 247]. This AKAP-dependent targeting may also be critical in any later receptor resensitisation [241]. Finally, it now appears that, in addition to desensitisation of $G_s$-coupled signalling (above), the recruitment of PDE4D5 *via* interaction with β-arrestin and AKAP79 (now AKAP5) is also critical in terminating the PKA-dependent switching of the $\beta_2$-adrenoceptor to $G_i$-dependent signalling down to ERK [238].

The other established process that promotes prolonged periods of desensitisation, and which may be of greater clinical relevance [2], is the downregulation of $\beta_2$-adrenoceptor number, during which physical internalisation and subsequent degradation of the receptor occurs [233]. This may involve inhibition of $\beta_2$-adrenoceptor transcription and/or increased post-transcriptional processing of $\beta_2$-adrenoceptor mRNA [233]. In addition to these well-characterised processes, scrutiny of studies published since the mid-1970s indicates that additional, and in some cases neglected, mechanisms could also play a major role in regulating $\beta_2$-adrenoceptor signalling and two of these are described below.

### *Upregulation of phosphodiesterase 4*

One mechanism that can contribute to desensitisation is the upregulation of one or more cAMP PDE isoenzymes [248]. This can occur through either post-translocational modification (*e.g.* phosphorylation) of existing enzyme or gene induction [249]. With respect to pulmonary $\beta_2$-adrenoceptor expression, it is the PDE4 isoenzyme family, which is encoded by four genes (PDE4A–PDE4D), that is a primary regulator of cAMP metabolism [250, 251]. In this paradigm, tolerance to $\beta_2$-adrenoceptor agonists is directly related to an increase in PDE activity. This effect would theoretically compromise cell signalling through all $G_s$-coupled receptors, leading to heterologous desensitisation of susceptible cells to cAMP-dependent events. It is hypothesised that this would occur as a direct consequence of regular treatment with $\beta_2$-adrenoceptor agonists. Significantly, this model does not exclude the participation of the other established mechanisms of desensitisation, described above. Indeed, phosphorylation of the $\beta_2$-adrenoceptor by GRKs and PKA could, theoretically, act in concert with cAMP PDE to limit the magnitude and duration of $\beta_2$-adrenoceptor-mediated signalling [248].

Although generally ignored, the concept of increased cAMP PDE activity as a mechanism of reducing the sensitivity of cells

to hormones and other agonists that interact with Gs-coupled receptors is not new. Indeed, evidence that this phenomenon accounts for much of the reduced responsiveness that cells exhibit to chronic hormone exposure was provided in 1978 [252], and has since been documented *in vitro* in many cells implicated in the pathogenesis of asthma, such as T-lymphocytes, neutrophils, monocytes, macrophages, platelets and ASM [154–157, 252–261]. Upregulation of PDE has also been demonstrated empirically in transfection experiments in which the engineered expression of cAMP PDE in yeast and mammalian cells reduces their sensitivity to hormones that augment AC activity [262–265].

An important issue that arises from the aforementioned discussion is whether or not induction and/or phosphorylation of PDE4 can be demonstrated in immune/pro-inflammatory cells and *in vivo* in response to $\beta_2$-adrenoceptor agonists. Although limited data are available, the answer to both parts of this question is yes. TORPHY *et al.* [154] demonstrated that the $\beta_2$-adrenoceptor agonist, salbutamol, and the selective PDE4 inhibitor, rolipram, when given in combination to the human monocytic cell line, U937, increased PDE4 activity in a time-dependent manner. Significantly, this effect required new protein synthesis, indicating that the increase in enzyme activity was attributable to the induction of one or more PDE4 isogenes. RT-PCR and Western blot analyses performed by the same authors demonstrated, subsequently, that salbutamol and rolipram increased the expression of PDE4A and PDE4B at both the mRNA and protein level [155]. A similar investigation by VERGHESE *et al.* [261] essentially confirmed these observations. Thus, exposure of human peripheral blood monocytes and Mono Mac 6 cells to cAMP-elevating agents promoted the transcription of the PDE4A, B and D isogenes, with the generation of at least three distinct mRNA transcripts and proteins. ENGELS *et al.* [266] have also reported induction of PDE4 isogenes in U937 and Jurkat T-cells in response to prolonged exposure to dibutyryl cAMP, and, more recently, the same phenomenon was documented in guinea pig macrophages [266], human T-lymphocytes [156], human neutrophils [256] and human ASM cells [157]. In the latter study, upregulation of the PDE4D5 splice variant was described, and this may be of particular significance given that this isoform interacts preferentially with β-arrestins and may play a role in $\beta_2$-adrenoceptor desensitisation (see above) [237, 238].

A consistent and highly significant finding is that the responsiveness of many cells in which PDE4 is induced to cAMP-generating agonists is restored, at least in part, by the addition of a PDE inhibitor, providing compelling evidence that upregulation of PDE is a significant contributory factor to the development of tolerance. In 2000, FINNEY *et al.* [267] reported the upregulation of PDE4 in the lungs of rats treated with salbutamol for 7 days. Thus, this phenomenon can be produced *in vivo* and may be of clinical relevance in the development of tolerance following long-term use of $\beta_2$-adrenoceptor agonists.

### *Downregulation of stimulatory G-protein subunit α*

Another poorly researched process that could promote heterologous desensitisation of Gs-coupled receptors is a reduction in the abundance of plasma membrane-bound Gsα [268]. FINNEY and co-workers [158, 267] have reported that long-term systemic treatment of rats with salbutamol and salmeterol blocks the ability of these agonists subsequently to protect against acetylcholine-induced bronchoconstriction. Moreover, the bronchoprotective effect of $PGE_2$, which also acts through Gs-coupled prostanoid receptors of the $PGE_2$ receptor 4 subtype in rat airways [269], was similarly abolished, indicating that a state of heterologous desensitisation had been effected. Significantly, further studies found that, in the lungs of rats treated with $\beta_2$-adrenoceptor agonists, there was a ~50% reduction in the level of Gsα and an associated impaired ability of cholera toxin to promote cAMP accumulation *ex vivo*.

## INTERACTION OF $\beta_2$-ADRENOCEPTORS WITH OTHER PROTEINS

A number of additional interactions have been described that extend the multiplicity of $\beta_2$-adrenoceptor-mediated responses, although none have yet been demonstrated in the airways. In particular, the $\beta_2$-adrenoceptor features a consensus PDZ domain at its carboxyl terminus that has been shown to interact in an agonist-dependent manner with the PDZ domain of the $Na^+/H^+$-exchanger regulatory factor (NHERF) [270]. In the absence of $\beta_2$-adrenoceptor agonist, NHERF binds to the type 3 $Na^+/H^+$ exchanger, thereby inhibiting pump activity [271]. However, this inhibitory activity is relieved in the presence of agonist, resulting in $\beta_2$-adrenoceptor-mediated activation of the $Na^+/H^+$ exchanger [270]. Evidence is also available that the NHERF plays a role in endocytic sorting of $\beta_2$-adrenoceptors [272]. Thus, when bound to NHERF, internalised $\beta_2$-adrenoceptors are recycled to the plasma membrane, whereas loss of this interaction results in lysosome targeting and receptor degradation [272].

$\beta_2$-Adrenoceptors have been shown to interact with at least two other proteins: the α subunit of eukaryotic initiation factor 2B [273], which is a nucleotide exchange factor that regulates mRNA translation, and *N*-ethylmaleimide-sensitive factor [274]. The former and latter interactions may have a role in regulating AC activity and $\beta_2$-adrenoceptor internalisation/recycling respectively.

## CONCLUDING REMARKS

According to PubMed records, cyclic adenosine monophosphate, since its discovery in 1958, is probably the second-most-studied second messenger, rivalled only by calcium, and has been implicated in a bewildering number of physiological and pathophysiological processes. It is, therefore, perhaps not surprising that the traditional dogma that activators of adenylyl cyclase, exemplified by agonists of the $\beta_2$-adrenoceptor, exert all of their effects by recruiting a single highly conserved pathway that involves the activation of protein kinase A and the subsequent phosphorylation of target proteins, has been discredited as the sole mechanism of action (fig. 1a). The recently appreciated diversity of $\beta_2$-adrenoceptor signalling, which is likely to evolve further, may offer clues as to the aetiology of some of the unwanted clinical effects elicited by $\beta_2$-adrenoceptor agonists and provide opportunities for the future development of novel and safer pharmaceuticals for the treatment of asthma and related respiratory diseases.

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