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REVIEW ARTICLE

ILLUMINATING ASTHMA'S COMPLEXITY: UNRAVELING THE MOLECULAR MECHANISM OF BETA-2 RECEPTOR AGONIST-DRIVEN BRONCHIAL SMOOTH MUSCLE RELAXATION

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Asthma is a common noncommunicable disease that causes airway inflammation, leading to airway hyperresponsiveness and airflow limitation due to reversible bronchoconstriction, increased mucus production, and complex airway inflammation. Despite this complexity, asthma treatments that include inhaling β_2-agonists are widely used. Two types of bronchodilators are commonly used to treat bronchial asthma and chronic obstructive lung disease including β_2-adrenoceptor agonists and theophylline. β_2-receptor agonists activate phosphorylation mechanisms, as well as NO donors activate bronchial KCa^{2+} directly. These compounds stimulate cAMP- and cGMP-dependent protein kinases, which phosphorylate and activate bronchial KCa^{2+}. Additionally, the role of exchange proteins directly activated by cAMP (Epac) in the relaxation of bronchial smooth muscle. Although β_2-receptor agonists are known to relax bronchial smooth muscle, the exact molecular mechanism remains unclear. Therefore, the present study aims to elaborate on this mechanism and provide a more comprehensive understanding of β_2-receptor agonist-mediated relaxation of bronchial smooth muscle in asthma.

Key words: β_2-adrenoceptor agonists, K_{ATP}, KCa^{2+}, cAMP, Salbutamol, Asthma.

INTRODUCTION

Asthma, a diverse airway inflammatory illness, is one of the most common diseases in developed countries. It is characterized by airway hyper-responsiveness, reversible airflow restriction due to bronchoconstriction, increased mucus production, and complex airway inflammation. Asthma is a frequent noncommunicable illness worldwide, affecting between 1 and 18 percent of the population in various nations, with a global prevalence of over 339 million [1]. Several studies suggest that the prevalence of asthma is higher in the United States than the global

average, with a higher percentage of children affected than adults. Asthma can be caused by various factors and triggering agents, including environmental factors, hormonal changes, allergies, irritants, infections, smoking, pregnancy, and vigorous exercise [2, 3].

Most mechanical force experiments involve applying mechanical loads, such as stretch, compression, and fluid flow shear stress, to cultured cells or tissue samples. Studies on human airway smooth muscle cells cultured in a fibronectin-coated silicon chamber showed that stretch-induced Ca^{2+} mobilization is not mediated by release from SR Ca^{2+} reserves but rather by a Ca^{2+} influx mechanism, possibly involving stretch-activated cation channels [4, 5]. Both Ca^{2+}-mobilizing and Ca^{2+}-independent processes mediate airway smooth muscle contraction, known as Ca^{2+} sensitization, specifically through the activation of the RhoA/Rho-kinase pathway in the asthmatic airway. Inositol-1,4,5-trisphosphate (IP3) binds to the IP3 receptor, triggering store-operated Ca^{2+} entry (SOCE), also known as capacitative Ca^{2+} entry, in response to a decrease in the SR's Ca^{2+} content [6].

Orai1 is a cation channel important for SOCE, and STIM1 is a crucial molecule that monitors Ca^{2+} concentrations inside the SR SOCE is activated when a ligand or agonist, such as a G-protein-coupled receptor, is bound to a receptor [7]. Some or all of the channels for SOCE may be made up of members of the transient receptor potential (TRP) protein family, specifically the canonical TRP (TRPC) subfamily, which are receptor-operated and store-operated channels leading to Ca^{2+} entry [8].

Pathogenesis of asthma

Asthma is a condition characterized by a heightened sensitivity of the airways, resulting in acute constriction in response to certain substances that do not cause such a response in unaffected individuals or an exaggerated narrowing of the airways in response to inhaled drugs [9]. This hyperresponsiveness can be demonstrated through the use of smooth-muscle-acting drugs like histamine or methacholine. Smooth muscle cells in the airways play a significant role in asthma in three ways. Firstly, they can be abnormally sensitive to external stimuli [10]. Secondly, airway wall thickening due to hypertrophy and hyperplasia can affect airway reactivity. Thirdly, these cells can secrete chemokines and cytokines that promote airway inflammation and the survival of inflammatory cells, especially mast cells. Calcium (Ca^{2+}), a ubiquitous second messenger, regulates many cellular activities, including proliferation, migration, contraction, and metabolism. Mitochondria have also been shown to store Ca^{2+} via uniporter and release it via the Na^{+}/Ca^{2+} exchanger, making them important Ca^{2+} signaling organelles [11, 12]. Both elevated extracellular Ca^{2+} and the release of intracellularly stored Ca^{2+} have been implicated in initiating the contractile mechanism of airway smooth muscle in asthma [13].

Moreover, studies investigating the contraction of airway smooth muscle have indicated that the majority of calcium influx in this muscle type originates from intracellular stores rather than from L-type voltage-gated calcium channels (L-VOCCs) [14]. Cytosolic calcium ions serve as second messengers in most signaling pathways, and an increase in their concentration triggers contractility in airway smooth muscle cells by stimulating calmodulin-dependent myosin light-chain kinase [15]. While excessive calcium ion concentration in the cytosol activates L-VOCCs in airway smooth muscle cells via membrane depolarization, this does not fully explain the occurrence of asthma. Furthermore, the increase in cytosolic calcium ions also stimulates the activity of migratory, proliferative, and inflammatory cytokines in airway smooth muscle cells [16] (**Figure 1**).

The KCa^{2+} channels, which are a diverse family of ion channels, exhibit a broad spectrum of biophysical and pharmacological characteristics. Despite this variability, these channels share a common function of coupling elevated intracellular Ca^{2+} concentrations with plasma membrane hyperpolarization [17]. This fundamental characteristic of KCa^{2+} channels allows them to play a crucial role in regulating cellular excitability, K^{+} homeostasis, and cell volume in non-excitable cells. The KCa^{2+} family can be classified into two subfamilies including the small conductance potassium channels (S.K.; KCa^{2+} 2.1, 2.2, 2.3), intermediate conductance potassium channels (I.K.; KCa^{2+} 3.1), and the big conductance potassium channels (B.K.; KCa^{2+} 1.1) [18, 19]. Unlike S.K. and I.K. channels, which are only activated by low cytosolic calcium, B.K. channels are allosterically activated in response to changes in membrane voltage or intracellular Ca^{2+} [20]. The wide range of KCa^{2+} channel properties observed across different tissues is influenced by several factors, including Ca^{2+}

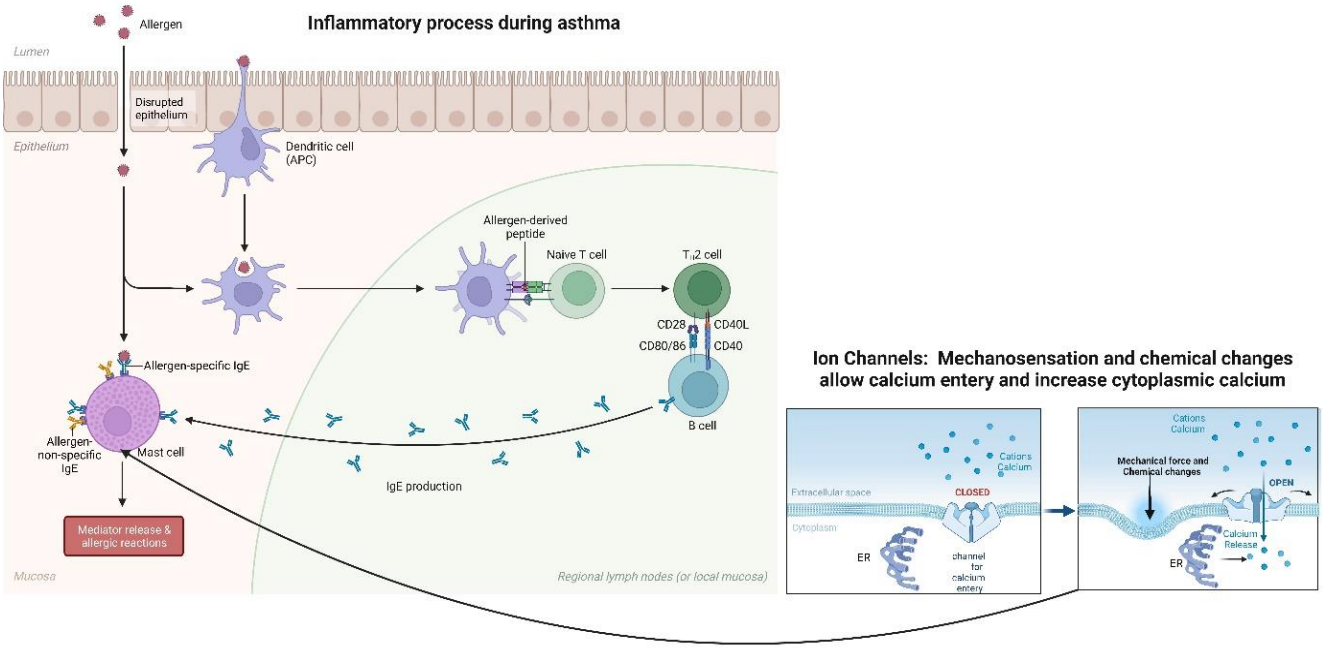

Fig. 1. Exhibiting the involvement of calcium associated release of inflammatory mediators in pathophysiology of asthma

sensitivity, kinetic behavior, susceptibility to pharmacological agents, and responsiveness to protein kinase activation [21].

Abnormal Ca^{2+} homeostasis may lead to increased airway reactivity and the release of chemical mediators from leukocytes, mast cells, and other cell types in individuals with asthma [22]. Calcium is a crucial mediator for the various neurotransmitters and physical stimuli that trigger bronchial constriction and mast cell mediator release, and a malfunction in cellular calcium regulation may be linked to the development of bronchial hyperreactivity in asthma. During the disease, excessive calcium influx can bind to calmodulin or a calmodulin-like regulatory protein in mast cells, as well as promote the activation of enzymes necessary for producing newly synthesized mediators such as histamine, prostaglandins, leukotrienes, and TNF-α [23, 24].

According to recent pathological findings, administering CCB therapy for 12 months can inhibit the remodeling of bronchial smooth muscle. In a mouse model of asthma, treatment with nicardipine reduced the levels of interleukin IL-4 and IL-5 in bronchoalveolar lavage fluid, as well as eosinophil counts, suggesting that CCBs may have anti-inflammatory and antifibrotic effects on TH2 inflammation. In a mouse model of pulmonary fibrosis, felodipine was found to inhibit the development of TGF-1-dependent myofibroblasts and collagen synthesis [25, 26]. These findings suggest that CCBs may slow the yearly progression of pulmonary function in asthma patients, making them a promising treatment option for asthma. While broncho-dilatory drugs can raise intracellular cAMP in airway smooth muscle and relax precontracted preparations post-agonist administration, these drugs are less effective in inhibiting the agonist-induced contraction pre-agonist administration [27-29].

Pharmacology of Salbutamol

Bronchial asthma and chronic obstructive lung disease are commonly treated with two types of bronchodilators including β_2-adrenoceptor agonists (β_2-receptor agonists) and theophylline. Although muscarinic receptor antagonists (tiotropium) are also bronchodilators, they are not relevant to the current investigation [30, 31]. These bronchodilators exert their broncho-dilatory action by increasing cAMP levels inside bronchial smooth muscles. β_2-receptor agonists activate the adenylyl cyclase enzymes, which catalyze the generation of cAMP, whereas β_2-receptor agonists like theophylline do so by competitively inhibiting the phosphodiesterase (PDEs) that hydrolyze cAMP [32]. The bronchodilator impact of short-acting β_2-receptor agonists (like salbutamol) accounts for their therapeutic efficacy, although theophylline

has been shown to possess additional significant anti-inflammatory properties. However, there are few preclinical and clinical studies that explain the phosphorylation mechanisms of β_2-receptor agonists [33, 34].

Several studies have explored different compounds' effects on bronchial KCa^{2+} activation. For example, NO donors have been found to stimulate cAMP- and cGMP-dependent protein kinases, which in turn phosphorylate and activate bronchial KCa^{2+} directly in vascular and airway smooth muscles [35]. Other studies have shown the role of exchange proteins directly activated by cAMP (Epac) in bronchial KCa^{2+} activation. However, the molecular mechanism of β_2-receptor agonists mediated relaxation of bronchial smooth muscle is not fully understood [36, 37].

For more than half a century, researchers have been speculating about the potential harmful effects of adrenergic bronchodilators (Long-acting beta-agonists, LABAs) in individuals with asthma. The mechanism causing these unfavorable clinical consequences remains unknown [38]. This debate aims to shed light on bronchodilator and bronchoprotective tolerance, which has been observed most frequently with short-acting medications but has also been shown to occur more quickly and to a greater extent with LABAs. Although the benefits of β_2-receptor agonists are well established, their use in asthma has been met with persistent safety concerns [39]. Human pancreatic beta and mast cells express β_2-adrenoreceptors. Beta2-receptor agonists, both short-acting and long-acting, can suppress mast cells by inhibiting calcium-mediated exocytosis and the release of histamine and leukotrienes to varying degrees, depending on the density of β2-receptors in the preparation [40]. Salbutamol's activities as a β_2-receptor agonist on pancreatic beta cells increase insulin secretion, but other processes, such as increased glucagon secretion and hepatic effects, cause a rise in blood glucose and an apparent loss in insulin sensitivity. It is worth noting that the activation of β_2-receptors (less depletion or change in ADP/ATP ratio in pancreases compared to the smooth muscle of lungs) and insulin secretion may result from the depolarization of beta pancreatic cells, which is primarily mediated by K_{ATP} inhibition [41, 42].

β_2-agonists were found to further increase the production of interleukin-6 by rhinovirus (RV), which is concerning as this cytokine has proinflammatory properties in the context of inappropriate use of β2-receptor agonists during asthma exacerbations [43]. Studies on the IL-6 promoter have revealed that β2- receptor agonists enhance RV-induced IL-6 production by binding to a cAMP response element (CRE). This suggests that the induction of IL-6 in bronchial epithelial cells occurs through the same mechanism (cAMP induction) as the positive effects observed in smooth muscle cells (SMCs) [44]. Some studies have explored the connection between β_2AR-cAMP-Epac1-dependent up-regulation of angiogenesis and found that β_2AR- and AC-activation, but not Epac1 stimulation, increased vascular endothelial growth factor (VEGF) production in human umbilical vein endothelial cells (HUVEC) [45, 46].

On the contrary, inhibiting KCa^{2+} pharmacologically resulted in elevated levels of IL-10 expression in peripheral Treg cells, as well as increased expression of IL-10's transcriptional regulators (KLF4, EBP4, and Blimp-1) [47]. This suggests that KCa^{2+} suppression may have potential therapeutic benefits for chronic inflammatory conditions such as inflammatory bowel disease. However, both the pathogenesis and treatment strategies involve an overload of cytoplasmic free calcium ions. Additionally, it remains unclear why calcium overload is stimulated by asthma therapy, which activates KCa^{2+}, but not during the pathology of asthma itself [48, 49].

Concept of ATP-sensitive potassium channel (K_{ATP})

K_{ATP} openers have been shown to significantly relax the smooth muscles of both guinea pig and human airways. However, a study on the relaxation induced by β_2-receptor agonists and NO-donors found that it is not affected by sulfonylurea-derived K_{ATP} blockers such as glibenclamide, indicating that K_{ATP} opening is not involved in the relaxation response to these drugs [50]. Conversely, other studies have explored the role of K_{ATP} by blocking the bronchodilatory effects of salbutamol with glibenclamide. These contradictory results highlight the need to understand the structural basis and binding affinity of drugs towards K_{ATP} in order to comprehend the molecular mechanism of K_{ATP} in conjunction with β_2-receptor agonists [51].

K_{ATP} channels play a crucial role in energy sensing across a broad range of bodily functions, making them effective therapeutic targets in human clinical trials. Activators of K_{ATP}, such as

nicorandil and pinacidil, have been used for decades to manage hypoglycemia and high blood pressure, while inhibitors such as glyburide and repaglinide are used to treat diabetes [52]. These compounds bind to distinct locations in the transmembrane region of the sulfonylurea receptor (SUR) subunit. K_{ATP} channels allow for potassium currents to pass through the cell membrane, with ATP acting as an inhibitor and ADP acting as an activator [53]. The conductance of potassium through K_{ATP} channels is regulated in response to fluctuations in the cellular levels of ATP and ADP. The therapeutic use of K_{ATP} activators and inhibitors is widespread in managing high blood pressure, angina pectoris, and type 2 diabetes [54].

K_{ATP} channels are composed of eight proteins, which include four inward-rectifier potassium channel 6 (Kir6) subunits that form the pore and four regulatory SUR subunits. The ATP-binding site is situated on the central Kir6 subunit, whereas the peripheral SUR subunit contains the activating Mg-ADP binding site [55]. There are three primary isoforms of SUR proteins, including the NBD-separated inward-facing orientation that causes inactivation mediated by insulin secretagogues, and the NBD-dimerized occluded orientation associated with KATP excitation, which is activated by K_{ATP} openers and synergizes with Mg-ADP. The K_{ATP} channel contains two SUR sites, SUR1 and SUR2, which are useful for developing inhibitors that trigger the depolarization of β cells and insulin secretion by binding to SUR1 [56]. Conversely, activators that open KATP via SUR2 sites help in passing the sodium current to the membrane, which lowers blood pressure and is used to treat hypoglycemia [57].

In a recent *in vivo* study, both SG-209 (2-nicotinamide ethyl acetate) and CNP, which are direct activators of the KATP channel, were found to equally enhance angiogenesis in the chick chorioallantoic membrane (CAM) [58]. However, both glibenclamide and 5-hydroxy decanoate (5-HD), which are K_{ATP} inhibitors, suppressed both the baseline and CNP-induced CAM angiogenesis. *In vitro*, the polypeptides VEGF and CNP, as well as the direct K_{ATP} openers nicorandil and SG-209, stimulated the expansion and migration [59].

The molecular mechanism of K_{ATP} challenging salbutamol mediated activation

Salbutamol and levalbuterol are two SABAs that are extensively used in the Americas and act as agonists at the β_2AR to increase KCa^{2+} levels. Salbutamol was first introduced for commercial sale in 1969 and remains a vital emergency treatment for individuals with asthma [60].

In the study of GPCRs, the β_2AR is often used as a surrogate for the receptor under investigation. The hamster β_2AR was the first GPCR to be successfully cloned in the late 20th century, and our understanding of GPCR pharmacology and signaling has significantly improved as a result of observations made from studying the β_2AR [61]. When endogenous agonists like adrenaline and norepinephrine or synthetic agonists like salbutamol, a β_2-agonist medication, activate the β_2AR, it couples with and activates the stimulatory Gs protein, resulting in the release of the subunit of Gαs in the canonical receptor signaling scenario. Gαs, once bound, activates adenylyl cyclase, which catalyzes the conversion of ATP to cAMP [62].

The balance between adenylyl cyclase (AC) synthesis and phosphodiesterase (PDE) breakdown tightly regulates the amount of cAMP in cells, with ten AC isoforms and eight PDE families known to function on cAMP. Heterotrimeric G-protein-coupled receptors (GPCRs) linked to Gαs (stimulatory) and Gαi (inhibitory) primarily provide upstream signals for the cAMP-PKA cascade on the cell surface [51, 64]. GNAI 1/2/3 encodes Gαi, whereas GNAS encodes Gαs. β_2 agonists and other extracellular ligands can influence GPCR activity, which eventually regulates their intracellularly linked G proteins' activation. Heterotrimeric G proteins, composed of α, β, γ, and subunits, each have several isoforms, including four major G families (Gαs, Gαi, Gαq, and Gα12/13). Upon activation, G proteins separate from the receptor and can activate downstream effectors. Membrane-bound AC isoforms are mainly regulated by Gαs and Gαi (AC1-9) [46, 65]. Additionally, specific isoforms (AC2/4/7) can be activated by Gβγ, and negative regulation of cyclase activity can commence through phosphorylation by PKA for AC5 and AC6. Notably, physiological Ca^{2+} levels can activate (AC1/3/8 *via* calmodulin) or inhibit (AC5/6) specific AC isoforms. Soluble AC (encoded by ADCY10), unlike other isoforms, remains in the cytoplasm and mitochondrial matrix, responding to calcium and bicarbonate changes [66].

The role of PKA in the airway relaxation mediated by β_2-agonists is still a matter of debate. One study suggested that PKA is not necessary for the inhibition of acetylcholine-

induced contractions by β_2-agonists. However, another study indicated that PKA is required for the β_2-agonist-induced airway relaxation. In airway smooth muscle cells, PKA has been shown to phosphorylate calcium-gated potassium channels, myosin light chain kinase, and activate Epac proteins in asthma [66] (**Table 1**).

Table 1. Acute and chronic administration of salbutamol associated dose dependent responses

S. No.	Pharmacological Responses	Mechanism of salbutamol
1	Bronchial smooth muscle cells relax	SUR 1 and 2 unit of K_{ATP} channel opening (activation) due to over utilization of ATP mediated enhanced mitochondrial phosphorylation and enhanced ADP through salbutamol at therapeutic range
2	Hyperglycemia	Changes in ADP/ATP ratio associated K_{ATP} channel opening at SUR 1 units leads to reduced insulin secretion in pancreatic beta cells
3	Cardiac arrhythmias	Higher dose of salbutamol changes the generation pattern of ADP and increase ATP/ ADP ratio mediated NBD-separated inward-facing orientation reflects an inactivated state of SUR2 unit of K_{ATP} in cardiac cell.
4	Seizures	Over dose of salbutamol associated neuronal depolarization due to inactivation of K_{ATP} at SUR 1 and SUR 2 unit

Our observations reveal that the Kir6.2 cytoplasmic domain can be either closely linked or extended into the cytoplasm depending on the presence of pharmacological inhibitors and ATP. The K_{ATP} channel is primarily regulated by the ATP/ADP ratio, where an increase in the ratio leads to channel closure, and a decrease in the ratio in the presence of Mg^{2+} ions leads to channel opening [3, 13]. The catalytic region formed by the Walker motifs is responsible for binding adenine nucleotides and hydrolyzing ATP to produce ADP, which is critical for maintaining proper K_{ATP} channel activity through ADP-induced K_{ATP} channel activation. β_2-agonists can increase human resting metabolic rates by 10-50%, and changes in nucleotides ATP to cAMP decrease the ATP/ADP ratio, as is seen with beta 2 receptor agonists like salbutamol [32, 37]. However, the activation of β_2 receptors by salbutamol can lead to hyperglycemia due to the K_{ATP}-associated opening of depleted insulin secretion from the pancreas and overproduction of glucose through gluconeogenesis and glycogenolysis in the liver. Research indicates that the K_{ATP} opener bepridil prevents Ca^{2+} overloading in guinea pig cells and their organelles, such as mitochondria. Notably, certain inhibitory isoforms (AC 2/4/7) can be activated by the Gβγ subunit of G-proteins, and the lung protein is overactivated due to calcium-mediated inhibition of AC5/6. Inhibiting the K_{ATP} pathway increases NLRP3 inflammasome and inflammatory mediators such as IL-6, MPO, TNF-α, and MCP-1, negating the anti-inflammatory effects of *Panax notoginseng* saponins [41, 48]. This finding was supported by the use of pharmacological blockers of K_{ATP}, such as 5-hydroxydecanoate (a selective inhibitor of mitoKATP) and glibenclamide (a nonselective inhibitor of KATP). Through the activation of β_2 adrenergic receptors, salbutamol can suppress both short-term and long-term inflammation by decreasing inflammatory mediators such as MPO, TNF-α, and IL-6. Given that inflammation contributes to the etiology of asthma, the anti-inflammatory effects of salbutamol may occur due to the opening of K_{ATP} and could be beneficial in treating the disease [24, 54, 62] (**Figure 2**).

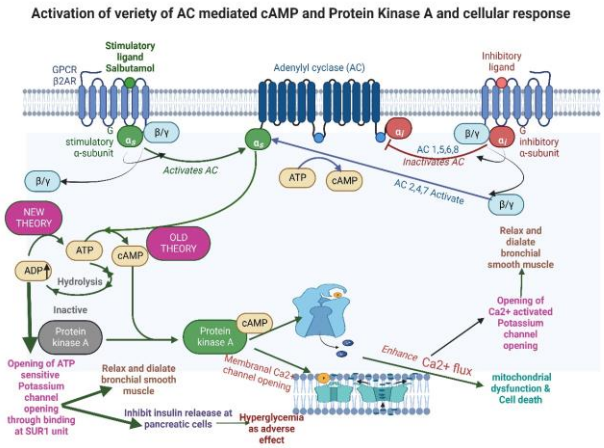

Fig. 2. Exploring mechanism of K_{ATP} challenging salbutamol mediated activation of KCa^{2+}

CONCLUSION

The latest advancements in studying the structure of K_{ATP}-drug complexes involving salbutamol have focus on the role of K_{ATP} pharmacology in treating asthma. Nevertheless, additional investigations are necessary to

comprehensively comprehend the molecular basis of K_{ATP} pharmacology in respiratory disorders.

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