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Inhibitors of *Bacillus anthracis* edema factor

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ABSTRACT

Edema factor (EF) is a calmodulin (CaM)-activated adenylyl cyclase (AC) toxin from *Bacillus anthracis* that contributes to anthrax pathogenesis. Anthrax is an important medical problem, but treatment of *B. anthracis* infections is still unsatisfying. Thus, selective EF inhibitors could be valuable drugs in the treatment of anthrax infection, most importantly shock. The catalytic site of EF, the EF/CaM interaction site and allosteric sites constitute potential drug targets. To this end, most efforts have been directed towards targeting the catalytic site. A major challenge in the field is to obtain compounds with high selectivity for AC toxins relative to mammalian membranous ACs (mACs). 3′-(N-methyl)anthraniloyl-2′-deoxyadenosine-5′-triphosphate is the most potent EF inhibitor known so far (K_i, 10 nM), but selectivity relative to mACs needs to be improved (currently ~5–50-fold, depending on the specific mAC isoform considered). AC toxin inhibitors can be identified in virtual screening studies based on available EF crystal structures and examined in cellular test systems or at the level of purified toxin using classic radioisotopic or non-radioactive fluorescence assays. Binding of certain MANT-nucleotides to AC toxins elicits large direct fluorescence- or fluorescence resonance energy transfer signals upon interaction with CaM, and these signals can be used to identify toxin inhibitors in competition binding studies. Collectively, potent EF inhibitors are available, but before they can be used clinically, selectivity against mACs must be improved. However, several methodological approaches, complementing each other, are now available to direct the development of potent, selective, orally applicable and clinically useful EF inhibitors.

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Contents

1. Introduction

cAMP is a ubiquitous intracellular second messenger that regulates numerous cell functions including energy metabolism, contractility, secretion, host defense, endocrine function and neuronal memory formation (Sunahara and Taussig, 2002; Lohse et al., 2003; Bender & Beavo, 2006; Rehmann et al., 2007; Taylor et al., 2008; Sadana & Dessauer, 2009). The cellular concentration of cAMP is regulated by extracellular first messenger molecules that include hormones, neurotransmitters and local mediators (Sunahara and Taussig, 2002; Sadana & Dessauer, 2009). Fig. 1 provides an overview on cAMP-mediated signal transduction, pharmacological interventions and interfaces of important bacterial toxins with cAMP signaling. A prototoypical first messenger is the hormone adrenaline that binds to heptahelical β-adrenergic receptors. These receptors activate the stimulatory G-protein of membranous AC, G_s. Mammals express nine membranous

Abbreviations: ANT, anthraniloyl; AC, adenylyl cyclase; ACD, adenylyl cyclase domain; EF, edema factor AC toxin from *Bacillus anthracis*; CaM, calmodulin; FRET, fluorescence resonance energy transfer; mAC, membranous mammalian AC; MANT, (N-methyl) anthraniloyl; NTP, nucleoside 5′-triphosphate; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; PMEApp, 9-[2-(phosphonomethoxy)ethyl]adenine diphosphate; TbNflx, terbium norfloxacin.

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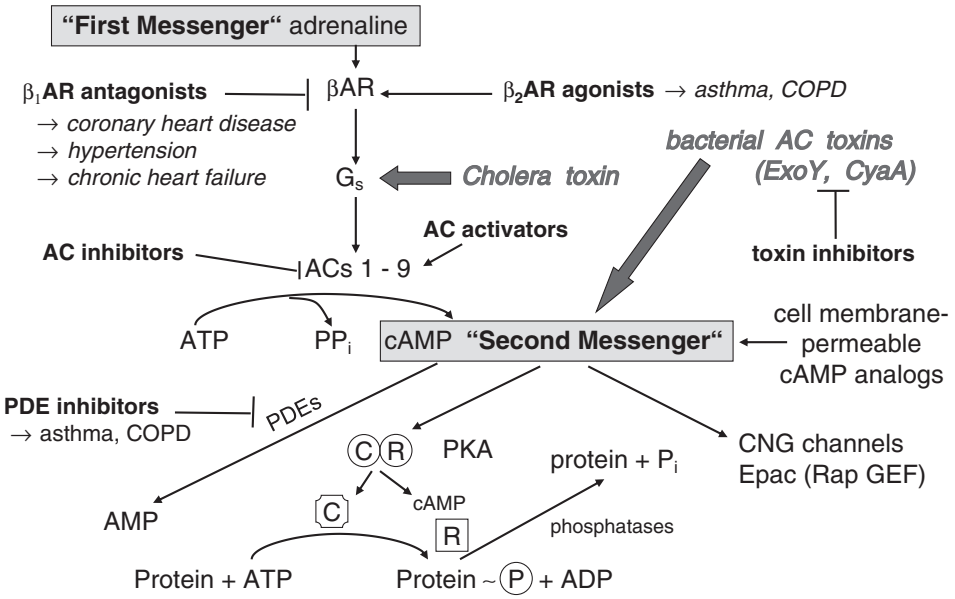

Fig. 1. Overview on cAMP-mediated signal transduction in mammalian cells, excessive activation of cAMP signaling by bacterial toxins and pharmacological interventions. C, catalytic subunit of cAMP-dependent protein kinase (PKA); R, regulatory subunit of cAMP-dependent protein kinase; CNG channels, cyclic nucleotide-regulated ion channels; COPD, chronic obstructive pulmonary disease; βAR, β-adrenergic receptors; Epac, guanine nucleotide exchange factor for the small GTP-binding protein Rap; PDEs, phosphodiesterases. For further explanations, see body text.

AC isoforms (ACs 1–9) and a soluble AC (Sunahara & Taussig, 2002; Sadana & Dessauer, 2009; Tresguerres et al., 2011). ACs catalyze the cyclization of ATP to cAMP. cAMP binds to, and alters the function of, cyclic nucleotide-gated ion channels and specific guanine nucleotide exchange factors for small GTP-binding proteins (Rehmann et al., 2007; Taylor et al., 2008). The best-studied intracellular target of cAMP is PKA (Taylor et al., 2008). cAMP binds to the regulatory subunit of PKA which then dissociates from the catalytic subunit. The catalytic subunit phosphorylates target proteins, changing their function and ultimately resulting in a cell type-specific response. Protein phosphatases dephosphorylate proteins and, thereby, reset the system. cAMP itself is degraded to AMP by PDEs, a complex superfamily of enzymes consisting of 11 different families (Bender & Beavo, 2006). Cell membrane-permeable cAMP analogs mimic the effects of cAMP generated by ACs (Schwede et al., 2000).

Several clinically important drugs change the function of cAMP signaling. β_2-adrenergic receptor agonists are used for the treatment of bronchial asthma and chronic obstructive pulmonary disease as are PDE inhibitors, both raising cAMP concentrations (Fig. 1). Chronic activation of the cardiac β_1-adrenergic receptor is deleterious (Lohse et al., 2003). Therefore, β_1AR antagonists are most useful drugs for the treatment of major cardiovascular diseases including coronary heart disease, hypertension and chronic heart failure. The diterpene forskolin from the Indian plant, *Coleus forskohlii*, activates AC isoforms 1–8 (Sunahara & Taussig, 2002; Sadana & Dessauer, 2009), but it has been difficult to implement the use of forskolin clinically because it is not sufficiently AC isoform-selective (Alasbahi & Melzig, 2012; Seifert et al., 2012). Topical application in target organs may be a strategy to avoid unwanted effects of forskolin (Wagh et al., 2012). In addition, recently, the fluorescent forskolin derivative BODIPY-forskolin has been revealed as an inhibitor of AC2 while being a partial activator of ACs 1 and 5 (Erdorf et al., 2011). Thus, the first step towards the development of diterpenes with selectivity for defined AC isoforms has been taken. There is current interest in the design of AC5 inhibitors for the treatment of aging and heart failure (Iwatsubo et al., 2012), but compounds with high potency and AC5 selectivity are very difficult to develop and not yet available (Pinto et al., 2011; Seifert et al., 2012; Bräunig et al., 2013). In addition, a recent study casts doubt about the supposed major contribution of AC5 to total AC activity in the heart (Bräunig et al., 2013). The literature on inhibitors for membranous ACs has been recently reviewed (Seifert et al., 2012).

2. EF as bacterial AC toxin

Several bacterial toxins exploit cAMP signaling very effectively (Fig. 1). Cholera toxin from the enteropathogenic bacterium *Vibrio cholerae* ADP-ribosylates the α-subunit of G_s and, thereby, blocks its GTPase activity (Moss & Vaughan, 1988). As a result, $G_{s\alpha}$ is permanently locked in its active GTP-bound state, resulting in uncontrolled cAMP formation. The massively increased cAMP production leads to profound gastrointestinal secretion, water and electrolyte loss, dehydration and, ultimately, death. cAMP-increasing substances also effectively reduce the function of various cell types of the immune system including phagocytes and T cells (Peters-Golden, 2009; Mosenden & Taskén, 2011). The bacterial AC toxins EF from *Bacillus anthracis*, the causative agent of anthrax infection, and CyaA from *Bordetalla pertussis*, the causative agent of whooping cough, exploit this mechanism to compromise host defense and facilitate propagation of the bacterial infection (Ahuja et al., 2004; Vojtova et al., 2006; Tang & Guo, 2009; Seifert & Dove, 2012).

Anthrax infection constitutes a very important medical problem. Most well known in the public are the 2001 anthrax bioterrorism attacks in the United States, but prevention of such attacks is still an unresolved public health and security issue (Danzig, 2012; Russell & Gronvall, 2012). Another important current problem is anthrax infection users of injection drugs such as heroine (Sweeney et al., 2011; Hicks et al., 2012). Anthrax in pregnant and postpartum women is clinically relevant, too (Meaney-Delman et al., 2012). Anthrax is also an important zoonosis in many parts of the world, but disease awareness is low (Beyer & Turnbull, 2009; John et al., 2011). Moreover, vaccination against *B. anthracis* and antibiotic treatment of anthrax infections are unsatisfying (Klinman et al., 2009; Doganay et al., 2010). Most importantly in the context of this review is the fact that EF plays an important role in the development of anthrax-associated shock (Hicks et al., 2011a; Li et al., 2013). Hence, the development of potent and selective EF inhibitors constitutes a highly relevant therapeutic goal to improve the prognosis of anthrax patients.

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