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Calcium channels blockers inhibit the signal transduction through the AtoSC system in *Escherichia coli*

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ABSTRACT

Verapamil, diltiazem and nifedipine are Ca^{2+}-channel blockers used in cardiovascular diseases. We report here that the *Escherichia coli* AtoSC signaling is inhibited by those blockers. AtoSC two-component system plays a pivotal role in sophisticated signaling networks in *E. coli* regulating processes implicated in bacterial homeostasis and pathogenicity. The Ca^{2+}-channel blockers abrogated the *in vitro* full-length AtoS kinase autophosphorylation. However, they demonstrated no effect on the AtoS cytoplasmic form autophosphorylation. AtoC protected AtoS from verapamil or diltiazem but not from nifedipine, when the two constituents formed complex. The blockers did not affect the AtoS∼P to AtoC phosphotransfer. The blockers-mediated AtoSC inhibition was verified *in vivo* on the *atoDAEB* expression, which was inhibited only in AtoSC-expressing bacteria upon acetoacetate. The AtoS and AtoC protein or their genes transcription levels were unaffected by the blockers. Blockers demonstrated differential effects in the regulation of both the cytosolic- and most potently the membrane-bound-cPHB. Extracellular Ca^{2+} counteracted the verapamil-mediated effect on cPHB only in *atoSC*$^+$ cells. Extracellular Ca^{2+} reversed the diltiazem-mediated cPHB decreases in cells of both genetic backgrounds, yet a Ca^{2+}-concentration dependent reversion was observed only in the AtoSC-regulated cPHB. Nifedipine caused a more pronounced cPHB down-regulation that was not reversed by extracellular Ca^{2+}. The AtoSC signaling inhibition by Ca^{2+}-channel blockers used for human treatment, and their differential effects on cPHB-formed Ca^{2+}-channels, signify their implications in bacterial–host interactions through the two-component signaling and could stimulate the design of Ca^{2+}-channels blockers derivatives acting as inhibitors of two-component systems.

1. Introduction

The implication of Ca^{2+} as a signal in a wide variety of cellular processes affecting the physiology and pathogenesis is now becoming evident of bacteria where cytosolic Ca^{2+} levels are tightly controlled by mechanisms involving plasma membrane transporters (Norris et al., 1996; Holland et al., 1999; Hoffer et al., 2001; Dominguez, 2004; Andersen et al., 2006). In *Escherichia coli*, Ca^{2+} influx arrangements include amongst others the non-proteinaceous complexes of short-chain poly-(R)-3-hydroxybutyrate-polyphosphate-Ca^{2+} (Reusch et al., 1995; Jones et al., 2003) acting as voltage-gated Ca^{2+} channels, implicated in the phase of growth-dependent control of cytosolic Ca^{2+} levels (Reusch et al., 1995; Campbell et al., 2007a, 2007b). The biosynthetic pathway of cPHB [complexed poly-(R)-3-hydroxybutyrate] is unidentified in *E. coli*, where a periplasmic protein, YdcS, has been reported to exhibit PHB synthase activity (Dai and Reusch, 2008) even though a number of physiological roles have been attributed to cPHB (Reusch et al., 1986, 1995; Xian et al., 2007).

The regulation of biosynthesis and the intracellular distribution of cPHB is mediated by the AtoSC two-component system (TCS) via its direct action on *atoDAEB* operon transcription (Theodorou et al., 2006, 2007) as well as according to recent studies through its involvement in fatty acids metabolism (Theodorou et al., 2011a). Adaptive microbial signal transduction involves a multi-faceted regulated phosphotransfer mechanism that comprises structural rearrangements of sensor histidine kinases (HKs) upon ligand-binding and phosphorylation-induced conformational changes in response regulators (RRs) of versatile TCS, arisen early in bacterial evolution (Kyriakidis and Tiligada, 2009). AtoSC TCS in *E. coli*, consisting of the AtoS HK and the AtoC RR plays a pivotal role in many regulatory indispensable processes, including also growth on short-chain fatty acids through the transcriptional activation of

Abbreviations: atoSC$^+$, genetic locus encoding the AtoS and AtoC proteins; AcAc, acetoacetate; [Ca^{2+}]e, extracellular Ca^{2+} concentration; cPHB, complexed poly-(R)-3-hydroxybutyrate; TCS, two component system; URR, upstream regulatory region.

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the *atoDAEB* operon, motility and chemotaxis regulation, some of them implicated to pathogenicity and bacterial–host symbiosis (Kyriakidis and Tiligada, 2009; Theodorou et al., 2011d). AtoSC-mediated signal transduction has been associated with acetoacetate (Theodorou et al., 2006), spermidine (Theodorou et al., 2007), or intermediate metabolic compounds of the short-chain fatty acid pathway (Theodorou et al., 2011c). It has been also correlated with the biogenic amine histamine and the mast cell degranulating calmodulin inhibitor compound 48/80 (C48/80) (Kyriakidis et al., 2008), possibly in an extracellular Ca^{2+}-mediated manner (Theodorou et al., 2009). The expression of AtoSC in some pathogenic *E. coli* strains as well as other pathogenic bacteria, led very recently to identification of three inhibitors of the AtoSC signaling, belonging to different chemical classes, i.e. Closantel, TNP-ATP and RWJ-49815 (Theodorou et al., 2011b).

The variability of cytosolic Ca^{2+} as a consequence of the external concentrations via cPHB channels can have marked effects on *E. coli* (Campbell et al., 2007a, 2007b). cPHB channels share characteristics with the eukaryotic L-type voltage-activated Ca^{2+} channels, including concentration-dependent block by lanthanum (Reusch et al., 1995) which are targeted selectively by the Ca^{2+}-channel blockers verapamil, diltiazem and nifedipine, despite their differences in pharmacological properties (McDonald et al., 1994; Ling, 1997; Andersen et al., 2006). Ca^{2+}-channel inhibitors phenylalkylamines (exemplified by verapamil), benzothiazepines (exemplified by diltiazem) and 4-aryl-1,4-dihydropyridines (exemplified by nifedipine), have been used extensively, both clinically and in research in eukaryotic systems in the treatment of various cardiovascular diseases and anticancer therapies (McDonald et al., 1994; Ling, 1997; Andersen et al., 2006). However, has been limited evidence for Ca^{2+} variations through the influx systems regulation in *E. coli*, generated by Ca^{2+}-channels blockers (Tisa et al., 2000; Hoffer et al., 2001; Jones et al., 2003; Dominguez, 2004; Theodorou et al., 2009), while a limited number of studies coincide with the functional implication of Ca^{2+} in regulating proinflammatory signals and for the cross-talk between Ca^{2+}-channel blockers and bacteria–host interactions (Tisa et al., 2000; Jones et al., 2003; Hung, 2007; Theodorou et al., 2009).

We have previously provided evidence for an underlying modulatory role of the AtoSC TCS along with Ca^{2+}-mediated homeostatic mechanisms (Theodorou et al., 2009), and in the present study we investigated the differential effects of the Ca^{2+}-channel blockers in the AtoSC TCS signaling and its regulatory action on cPHB biosynthesis, with potential implications in its role in bacterial physiology and possibly pathophysiology or bacteria–host communication.

2. Materials and methods

2.1. Materials

All chemicals were obtained from Sigma–Aldrich (Chemie GmbH, Steinheim, Germany) unless otherwise stated. Verapamil hydrochloride was from Knoll AG (Ludwigshafen, Germany). [γ-^{32}P]-ATP (6000 Ci/mmol) was purchased from ICN Pharmaceuticals France S.A. Molecular weight protein markers were obtained from Gibco-BRL (Karlsruhe, Germany).

2.2. Bacterial strains, plasmids and growth conditions

The genotypes of *E. coli* strains used are listed in Table 1. *E. coli* K-12 strains BW25113 (*atoSC*$^{+}$) and BW28878 (Δ*atoSC*) (Oshima et al., 2002) were a gift from Hirofumi Aiba (Nagoya University, Japan).

Plasmid pUC-Az, containing the *atoS* and *atoC* genes and a part of the *atoDAEB* operon was constructed as described previously (Canellakis et al., 1993). Plasmids pHis$_{10}$-AtoC, pET21b-AtoS and pET21b-cytoAtoS, expressing the recombinant His$_{10}$-AtoC, His$_6$-AtoS and His$_6$-cytoAtoS, the cytosolic form of AtoS (lacking the two N-terminal transmembrane domains) respectively, have been described (Lioliou et al., 2005; Filippou et al., 2008).

Plasmid pEMZ-patoD1 containing the *atoDAEB* URR fused to a promoterless *lacZ* gene (*atoD1-lacZ* in pDP8) has been described (Lioliou et al., 2005). Plasmid pCPG6 (*atoC-lacZ*) carries a 1.337 kb fragment, containing a part of *atoS* gene, the *atoC* promoter region and 187 bp downstream of +1 of *atoC* gene (−1150 to +187 bp region), fused to the promoterless *lacZ* gene on pMLB1034 vector. Plasmid pCPG4 (*atoS-lacZ*) carries a 0.918 kb fragment, containing the *atoS* promoter region and 189 bp downstream of +1 of *atoS* gene (−729 to +189 bp region), fused to the promoterless *lacZ* gene on pMLB1034 vector (Matta et al., 2007).

To determine cPHB, *E. coli* was grown at 37 °C in LB (Luria–Bertani) broth (Sambrook et al., 1989). The AtoSC TCS was induced by the addition of 10 mM acetoacetate (Theodorou et al., 2006, 2007). $CaCl_2$, the blockers (verapamil, diltiazem, nifedipine) or $LaCl_3$ (0.2 or 0.6 mM) were added in the culture medium when A_{600} reached 0.3. No significant variation in either growth rates or final cell densities was observed between the strains growing under the same conditions. Cell culture samples were collected at the time of the first addition of acetoacetate and subsequently at the specified time points indicated and processed for crotonic acid/cPHB determination.

E. coli BL21[DE3] transformed with pHis$_{10}$-AtoC, pET21b-AtoS or pET21b-cytoAtoS were grown at 37 °C in LB medium, for the purification of the His$_{10}$-AtoC, His$_6$-AtoS or His$_6$-cytoAtoS. Ampicillin was added in the culture media, at final concentration of 100 μg/ml. Induction of recombinant His$_{10}$-AtoC expression was achieved at 28 °C, while of His$_6$-AtoS or His$_6$-cytoAtoS at 25 °C (where it was observed that the proteins were maintained in the cytoplasmic fraction and not in inclusion bodies) by addition of IPTG (1 mM) to the cultures when A_{600} reached 0.4.

2.3. Purification of recombinant AtoS, cytoAtoS and AtoC protein forms under native conditions

The purification His$_{10}$-AtoC or His$_6$-AtoS from BL21[DE3] cells transformed with pHis$_{10}$-AtoC (Lioliou et al., 2005) or pET21b-AtoS (Filippou et al., 2008) plasmids, overexpressing the His$_{10}$-AtoC or His$_6$-AtoS recombinant forms, respectively, was peformed using affinity chromatography on Ni^{2+}-IDA columns (Protino® NI-IDA, Macherey–Nagel, Germany), as previously reported (Theodorou et al., 2011b). The purification of His$_6$-cytoAtoS, the cytosolic form of AtoS (lacking the two N-terminal transmembrane domains), from BL21[DE3] cells transformed with pET21b-cytoAtoS was preformed as previously reported (Filippou et al., 2008).

2.4. Protein determination and immunoblotting analyses

Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the reference standard. Cells were grown in the presence of blockers at the final concentrations demonstrating *in vitro* or *in vivo* effects on the *atoSCDAEB* regulon i.e. verapamil 500 μM or 2 mM, nifedipine 100 μM or 3 mM, or diltiazem 100 μM or 2.5 mM and culture aliquots of 1 ml were harvested when A_{600} reached 0.4 and subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE). SDS–PAGE was performed using 10% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS as described by Laemmli (1970). Gels were stained with either Coomassie Brilliant Blue R250 or silver nitrate