Cyclic nucleotide–gated (CNG) channels produce the initial electrical signal in mammalian vision and olfaction. They open in response to direct binding of cyclic nucleotide (cAMP or cGMP) to a cytoplasmic region of the channel. However, the conformational rearrangements occurring upon binding to produce pore opening (i.e. gating) are not well understood. StHK is a bacterial CNG channel that has the potential to serve as an ideal model for structure–function studies of gating but is currently limited by its toxicity, native cysteines, and low open probability ($P_o$). Here, we expressed StHK in giant Escherichia coli spheroplasts and performed patch-clamp recordings to characterize StHK gating in a bacterial membrane. We demonstrated that the $P_o$ in cAMP is higher than has been previously published and that cGMP acts as a weak partial StHK agonist. Additionally, we determined that StHK expression is toxic to E. coli because of gating by cytoplasmic cAMP. We overcame this toxicity by developing an adenylate cyclase–knockout E. coli cell line. Finally, we generated a cysteine-free StHK construct and introduced mutations that further increase the $P_o$ in cAMP. We propose that this StHK model will help elucidate the gating mechanism of CNG channels.

Cyclic nucleotide–gated (CNG)$^2$ channels mediate signal transduction in the visual and olfactory systems (1–3). Although they belong to the voltage-gated ion channel superfamily, CNG channels are only weakly voltage-dependent and instead open (gate) upon direct binding of cyclic nucleotide (cNMP) to a cytoplasmic region of the channel (4, 5). In this manner, CNG channels transduce changes in secondary mes-senger concentrations into alterations in membrane voltage and intracellular Ca$^{2+}$.

CNG channels assemble as a 4-fold symmetric tetramer with an ion-conducting pore along the 4-fold axis. Each subunit is composed of six transmembrane (TM) helices followed by a large cytoplasmic region (6–8). In the TM region, S1–S4 comprise the voltage-sensing domain, and S5 and S6 comprise the pore domain with the selectivity filter between S5 and S6 (Fig. 1A). Immediately following the TM region is a C-linker domain composed of six helices (A′–F′) followed by a cyclic nucleotide–binding domain (CNBD), which contains four helices (A–C and P) and a β-roll (Fig. 1A) (6–8). The C-linker region contains extensive intersubunit contacts with the A′ and B′ helices of one subunit resting upon the C′ and D′ helices of the adjacent subunit (clockwise if looking from the extracellular side) in an “elbow on the shoulder” configuration (Fig. 1A) (7, 9).

Like other allosteric proteins, CNG channels transmit a conformational change from the ligand-binding site in each subunit (CNBD) to an active site (pore gate) (Fig. 1A). This allosteric regulation can be envisioned in a gating scheme based on Monod, Wyman, and Changeux (MWC) (10) where a concerted channel opening transition is driven by an increase in affinity for the ligand when the pore gate is open. In this scheme, the opening conformational change of the channel in the absence of ligand is represented by the equilibrium constant $L_o$ (Fig. 1B). The dissociation constant ($K_{o}$) describes binding of ligand (a) to the closed conformation of a single subunit, and each binding event causes the opening equilibrium to increase by a factor (f). Hence, the fully liganded (four bound cAMP molecules) opening equilibrium constant ($L$) is equal to $L_o f^4$.

Microscopic reversibility dictates that the conformational change occurring in the CNBD increases the affinity for the ligand by the same factor, f. Therefore, the allosteric conformational change is energetically driven by a higher affinity for the ligand when the channel is in the open state ($K_{o} f$) relative to when it is in the closed state ($K_{o}$) (Fig. 1B) (10–12). For retinal CNG channels, cGMP and cAMP both bind with a similar affinity to the closed channel ($K_{o}$); however, cGMP binds with a much higher affinity to the open channel ($K_{o} f$). As a consequence, the open probability ($P_o$) in saturating cGMP is nearly 1, whereas the $P_o$ in saturating cAMP is between 0.01 and 0.1 (13, 14).

The conformational change occurring in the CNBD to produce an f-fold change in affinity and channel activation has been elucidated largely due to structural and biochemical...
**SthK characterization and optimization**

![Figure 1. SthK structure and allosteric gating scheme.](image)

A, cartoon representation of SthK cryo-EM structure (Protein Data Bank (PDB) code 6CJU) with the functional domains labeled. cAMP is displayed as spheres. PD, pore domain; VSD, voltage-sensing domain. B, MWC gating scheme with C and O representing closed and open states, respectively. cAMP is indicated as a.

Despite the detailed knowledge of the conformational change occurring in the CNBD, the conformational changes occurring in the C-linker and TM region are largely unknown due, in part, to the lack of a suitable biochemical model for a full-length CNG channel. Recently, a family of prokaryotic CNG (pCNG) channel homologues was discovered that could serve as a model for studying full-length CNG channels in a purified system (21, 22). However, several properties prevent pCNG channels from serving as an ideal model system for studying gating. First, SthK, the pCNG for which electrophysiology is most amenable, reportedly has a low $P_o$ except at very depolarized voltages. At 0 mV, the voltage for most structural experiments of purified protein, SthK has been shown to have an $\sim 10% P_o$ in saturating cAMP, thus limiting studies of the conformational transitions in the C-linker and TM region (22). Indeed, a recent structure of cAMP-bound SthK and a structure of another cAMP-bound pCNG, LliK, both show the channel in a closed state (8, 23). Second, expression of SthK is toxic to *Escherichia coli*, which limits the amount of channel protein that can be purified. Finally, a cysteine-free version of SthK is not currently available and is required for site-specific labeling through cysteine modification. Site-specific labeling is a valuable tool for studying conformational dynamics with cysteine modification/cross-linking, double electron–electron resonance spectroscopy, and fluorescence spectroscopy.

Here, we optimize SthK as a model system for studying CNG channel gating. We performed both macroscopic and single-channel patch-clamp recording of SthK expressed in bacterial spheroplasts. We characterized the cNMP dependence and voltage dependence of SthK in a bacterial membrane, developed a method to reduce the toxicity of SthK expression, and engineered a Cys-free SthK construct with a high $P_o$.

**Results**

**Expression and patch-clamp recording of SthK in giant *E. coli* spheroplasts**

Although it is often easy to express eukaryotic channels in mammalian cells or *Xenopus* oocytes and record ionic currents, many bacterial channels are not expressed at high levels in these systems. To overcome this problem, we have expressed SthK in *E. coli* and then converted the cells into giant spheroplasts for patch-clamp recording (24–26). *E. coli* expressing full-length WT SthK with a C-terminal GFP tag (wtSthK) were treated with the antibiotic cephalexin, which blocked the final stage of binary fission and generated long “snake-like” cells with an interconnected cytoplasm (Fig. 2A). Then the cell walls were degraded, allowing the cells to relax into spheroplasts, which are mainly composed of naked inner membrane and are amenable to patch-clamp recording. Expression of wtSthK was visualized by fluorescence of the C-terminal GFP tag (Fig. 2B).

To record wtSthK currents, we formed inside-out patches from wtSthK-expressing spheroplasts using the patch-clamp technique (27). In the absence of cNMP, most patches show no currents from intrinsic channels and a small, ohmic leak current (Fig. 2C, top). Perfusion of the cytoplasmic face of the patch with cAMP produced maximal ionic currents at $-120$ mV between 50 pA and 3 nA. With voltage steps, a small time-dependent increase in the current occurred upon stepping to depolarized potentials, and a current decline occurred upon stepping to hyperpolarized potentials (Fig. 2C). These kinetic features indicate a slight depolarization dependence to activation, which has previously been observed in SthK in *Xenopus* oocytes and artificial lipid bilayers (21, 22). Furthermore, inward currents were larger and noisier at hyperpolarized voltages, suggesting a larger single-channel conductance and lower $P_o$ at hyperpolarized potentials (Fig. 2C). Finally, perfusion of a solution containing cGMP elicited no macroscopic currents (Fig. 2C, bottom). These results indicate that spheroplast patch-clamp recording is a feasible method for studying wtSthK gating and permeation.
Cyclic nucleotide dependence of wtSthK in the bacterial membrane

We first measured the cAMP dependence of wtSthK in the bacterial membrane using macroscopic currents. By varying the concentration of cAMP perfused onto the patch and measuring the steady-state current evoked at −60 mV, we obtained a dose-response curve with a $K_{1/2}$ of 1.5 ± 0.4 μM and Hill coefficient (h) of 1.5 ± 0.1 (n = 3) (Fig. 3A). These values differ significantly from the $K_{1/2}$ of 17 μM and h of 3 reported in artificial bilayers and the $K_{1/2}$ of 3.7 μM and h of 1.3 reported in Xenopus oocytes (21, 22).

Previous studies have disagreed on whether cGMP acts as an inhibitor (i.e., f ≤ 1) or as a weak partial agonist (f is small but >1) (Fig. 1B) (21, 22). To determine the efficacy of cGMP activation of SthK, we held a patch containing 150–200 wtSthK channels at −60 mV and perfused it with 5 μM cGMP while continuously recording for ~1 min (Fig. 3B). On this time scale, a number of small inward current spikes were visible that were not seen in the absence of cyclic nucleotide (Fig. 3B). Zooming in on one of these spikes shows a clear 50–100-ms single-channel open burst (Fig. 3B). Idealizing the trace gave an estimate of $P_o$ in cGMP between $10^{-6}$ and $10^{-5}$. These results support the conclusion that cGMP acts as a very weak partial agonist, although likely with lower efficacy than shown previously (22). This conclusion is further supported by our results from mutant channels below.

It has previously been reported that SthK exhibits a slow component of the activation time course indicative of mode shift or hysteresis (21, 22). By measuring macroscopic currents in inside-out patches with a rapid perfusion system, we performed concentration-jump experiments to assess whether we could observe evidence of a slower conformational change. In a patch held at −40 mV, currents elicited by a jump from 0 to 1 mM cAMP (~1000× $K_{1/2}$) were fit to a single exponential with no sign of a slower component on the 1–10-s timescale (Fig. 3C). The ~10–15-ms time constant measured for these concentration jumps was likely limited by the rate of perfusion and does not accurately represent the true course of channel activation. However, our results show no sign of a slow component of activation at these saturating cAMP concentrations.

Voltage dependence of wtSthK in the bacterial membrane

We next characterized the voltage dependence of wtSthK in a bacterial membrane using macroscopic currents. We recorded currents in saturating cAMP in response to a family of voltage pulses and measured the steady-state conductance from the leak-subtracted instantaneous tail currents at +100 mV (Fig. 4A). The resulting normalized conductance–voltage ($G–V$) curve displayed a shallow sigmoidal shape with a minimal value of 0.58 at −120 mV, indicating that voltage can reduce the $P_o$ by ~40% of its maximal value but cannot completely close the channel (Fig. 4B). Fitting this $G–V$ curve with the Boltzmann equation yielded a $V_{1/2}$ of 22 ± 7.4 mV and an e-fold change every 46.6 mV (Fig. 4B). This weak voltage dependence corresponds to a δ of 0.55 ± 0.02 equivalent electronic charges (n = 3), similar to the value of 0.8 charges previously reported based on single-channel $P_o$ recordings in artificial bilayers (22). This $G–V$ curve suggests the channel opening transition (L) is voltage-independent but coupled to a voltage sensor that moves the equivalent of 0.5 electronic charges across the membrane per wtSthK tetramer.

To determine the $P_o$ more directly, we recorded single-channel currents at −60 and +60 mV. The current traces showed bursting behavior with longer closures between bursts and rapid flickering closures within a burst (Fig. 4, C and D). All-points histograms of the trace were fit with the sum of two Gaussians, indicating a $P_o$ of 0.61 ± 0.046 at −60 mV (n = 10) and 0.90 ± 0.018 at +60 mV (n = 5). This $P_o$ is consistent with our $G–V$ curve (Fig. 4B) and is substantially higher than what
has been previously reported for SthK in *Xenopus* oocytes or artificial membranes (21, 22).

A single-channel voltage ramp from −110 to +120 mV revealed a strong inward rectification. The amplitude for inward currents at hyperpolarized voltages was about twice as large as the outward currents at depolarized voltages (Fig. 4E). A 3-fold higher conductance was seen for inward currents at −100 mV compared with outward currents at +100 mV in artificial membranes (22). However, the single-channel conductance we observed was about 3- and 2-fold smaller for inward and outward currents, respectively (22). This effect could be due to a difference in the expression system, rapid block by the 20 mM Mg²⁺ in our recording solutions, or reduced ion diffusion caused by the 500 mM sucrose in our recording solutions.

**Generation and functional characterization of a cysteine-free SthK construct**

The ability to perform site-specific labeling of cysteine residues through thiol-reactive probes, such as maleimides, enables spectroscopic experiments such as fluorescence or electron paramagnetic resonance (EPR). To optimize the SthK construct as a biochemical model for studying CNG channel gating, we mutated the two native cysteines to generate a Cys-free SthK construct (cfSthK). Based on an alignment with other pCNG channels, Cys-153, located near the extracellular end of S5, was mutated to Val, and Cys-387, located on the β-roll >15 Å from the ligand-binding site, was mutated to Ser. The resulting cfSthK has very similar properties to wtSthK. Perfusion of cAMP produced large currents, whereas perfusion of cGMP did not produce measurable currents (Fig. 5A). Furthermore, a cAMP dose-response curve yielded values of $K_{1/2} = 1.1 \pm 0.1$ μM and $h = 1.5 \pm 0.04$ (n = 3), both of which are similar to wtSthK (Fig. 5B).

In addition, single channels of cfSthK also behave similarly to single channels of wtSthK. A single-channel recording at −60 mV in saturating cAMP again showed bursting behavior (Fig. 5C). Fitting an all-points histogram gave a $P_o$ of 0.50 ± 0.041 (n = 5). This cfSthK background served as the foundation for our subsequent experiments.

**Overcoming toxicity of SthK expression**

The ability to express and purify large quantities of protein is an important feature of a model system for structure–function studies. Unfortunately, expression of wtSthK and cfSthK was toxic to *E. coli*. When C43 cells containing cfSthK under an IPTG-inducible promoter were induced in mid-log phase, the OD₆₀₀ remained relatively constant over the next 10 h (Fig. 6A). This suggests that cfSthK expression either halts growth or kills cells at a similar rate as division, resulting in a constant steady-state density and low overall expression.

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**Figure 3. Cyclic nucleotide-dependent gating of wtSthK.** A, dose-response curve of wtSthK channels for cAMP. The **solid black line** represents fit of the Hill equation with $K_{1/2} = 1.5 \mu$M and $h = 1.5$. Points and error bars represent mean ± S.E., respectively, from three patches. B, single-channel currents from a patch containing 150–200 wtSthK channels held at −60 mV in the absence of cNMP (upper trace) and in the presence of 5 mM cGMP (lower trace). The inset shows zoom of an −1-s region. Data were filtered at 0.5 kHz for display. C, concentration jump from 0 to 1 mM cAMP while holding the patch at −40 mV. The inset shows an −500-ms region. The **thin red line** represents fit of a single exponential with a time constant of 11.0 ms.
We hypothesized that the toxicity of cfSthK expression is due to binding of cAMP and subsequent opening of the channel in the bacteria during expression. SthK has a higher apparent affinity (\(K_{1/2}\)) for cAMP than does \(E. coli\) cAMP receptor protein (\(K_{1/2}\)), suggesting that physiological cAMP concentrations could activate SthK during expression (28). To test this possibility, we mutated the conserved Arg-377. This Arg residue is found in the CNBD where it forms salt-bridge and hydrogen-bond interactions with the phosphate of cAMP (9, 29) (Fig. 6B). In CNG and HCN channels, mutation of this residue dramatically decreases the apparent affinity for cNMP (30, 31). When inside-out patches from \(E. coli\) expressing the cfSthK-R377Q mutant were perfused with 15 mM cAMP, substantial current was observed but with very large voltage-dependent current relaxation, suggesting that this cAMP concentration was not saturating (Fig. 6C). Indeed, perfusion with 1 mM cAMP produced only single openings (Fig. 6C, inset). These experiments demonstrate that mutation of Arg-377 dramatically decreased the apparent affinity for cAMP with a \(K_{1/2}\) shifted at least 1000-fold to a value greater than 1 mM. This \(K_{1/2}\)
for cfSthK-R377Q is likely to be outside the range of cAMP concentrations in *E. coli*. Interestingly, *E. coli* cells transformed with a plasmid carrying the more dramatic cfSthK-R377A mutant and induced at mid-log phase continue to grow to more than 3× the OD600 reached by cfSthK-expressing cells (Fig. 6A). This dramatic rescue suggests that the observed toxicity is indeed due to cAMP-dependent gating of SthK during expression.

Although mutation of Arg-377 presents an opportunity for producing cfSthK on a larger scale, the high cAMP concentration required to activate the cfSthK-R377A channels diminishes its usefulness as a model system for CNG channels. Therefore, we pursued an alternative strategy to reduce SthK toxicity by producing a strain of C43 *E. coli* cells lacking adenylate cyclase (*cyaA*), the enzyme responsible for cAMP synthesis. Deletion of *cyaA* from the C43 genome was accomplished using oligonucleotide-mediated recombination (32). Successful loss of *cyaA*, with replacement by the gene encoding chloramphenicol resistance (CAT), was confirmed by colony PCR using locus-specific primers (Fig. 6D). This new strain, termed C43 *cyaA−*, displayed a somewhat slower growing phenotype that was rescued by addition of cAMP to the growth medium, consistent with previous reports of *cyaA* disruption in *E. coli* (Fig. 6E) (33, 34).

To test for toxicity of cfSthK expression in the adenylate cyclase knockout, C43 *cyaA−* cells were transformed with either cfSthK or cfSthK-R377A, and expression was induced at mid-log phase. The growth of C43 cells expressing cfSthK was rescued by the *cyaA−* knockout, showing similar growth rates as well as similar final densities as C43 *cyaA−* cells expressing cfSthK-R377A (Fig. 6F). These results further indicate that SthK expression toxicity is indeed due to endogenous cAMP gating the channel during growth. More importantly, this strategy allows the production of large quantities of SthK with a high affinity for cAMP.

**Engineering a cfSthK construct with higher opening favorability**

Previous reports have shown that SthK has an energetically unfavorable opening transition (*I* in Fig. 1B) as indicated by a low *P*<sub>o</sub> at saturating CAMP concentrations, reported to be 0.14 at +100 mV and 0.65 at +200 mV (21, 22). As demonstrated earlier, SthK displays weak depolarization-dependent activation, indicating that the *P*<sub>o</sub> at 0 mV is substantially lower (Fig. 4B). Many structural experiments including cryoelectron microscopy (cryo-EM) and double electron–electron resonance spectroscopy are currently only feasible at 0 mV. Under these conditions, the ensemble of channels on an EM grid or in
a cuvet will assume an equilibrium distribution with a vast majority of StkK channels in the closed state (Fig. 1B). This point is most clearly illustrated in the recently published structure of StkK, which shows essentially identical closed conformations for the apo-, cAMP-bound, and cGMP-bound StkK channel (8). Although we observed a higher \( P_o \) of wtStkK in bacterial membranes than has been previously reported, our \( P_o \) estimate of 0.73 at 0 mV (Fig. 4B) suggests that alternative approaches may be necessary to study the opening conformational transition using purified StkK.

To increase the \( P_o \) for StkK, we made four mutations in the cfStkK background. On the D’ helix of the C-linker, Arg-284 and Glu-290 were both mutated to Gln (Fig. 7A, right) based on a report that mutation of the equivalent residues in bovine retinal CNG channels (bCNGA1) to the corresponding residues in the Caenorhabditis elegans CNG channel (TAX-4) substantially increases the efficacy of its partial agonist, cAMP (35). On the C-helix of the CNBD, Leu-422 was mutated to Gln based on a report that a similar mutation in bCNGA1 also increases cAMP efficacy (Fig. 7A, right) (19). Finally, Ala-208 on S6 was mutated to Val (Fig. 7A, left). This mutation was discovered serendipitously and found to increase maximal \( P_o \) in cfStkK. Incidentally, the equivalent residue in TAX-4 is also a Val (6). Then, the resulting construct contains three mutations to the corresponding residues in TAX-4, which has a very favorable opening transition and an open-channel cryo-EM structure (6, 35) We named this construct cfStkK-3QV to reflect the three mutations to Gln and the mutation to Val.

Strikingly, single-channel recording of the cfStkK-3QV construct revealed a very high \( P_o \) even at \(-60 \text{ mV} \) (Fig. 7B). Similar to wtStkK, the traces of cfStkK-3QV showed both longer-lived closures and very brief closures. However, the longer closures were much shorter than those observed in wtStkK and cfStkK (Figs. 4C and 5C). Fitting an all-points histogram gave a value of 0.92 ± 0.007 for \( P_o \) at \(-60 \text{ mV} \) (Fig. 7B).

To determine the extent by which the cfStkK-3QV construct increased favorability of the opening transition (\( L \) in Fig. 1B), we measured the activation by the weak partial agonist, cGMP, because \( P_o \) with a partial agonist is more sensitive to changes in \( L \) than \( P_o \) with a full agonist (36, 37). Of the four mutations added to cfStkK that comprise the cfStkK-3QV construct, L422Q is the only mutation of a residue in the CNBD that interacts with cNMP in the binding site (Fig. 7A) and, hence, might change agonist specificity. Therefore, we compared cfStkK-L422Q and StkK-3QV channels, which interact with the cAMP and cGMP through identical residues. Strikingly, the fractional activation by cGMP relative to cAMP at \(-60 \text{ mV} \) increases dramatically between these two channels, from 2.5 ± 0.57% for cfStkK-L422Q (\( n = 3 \)) to 89 ± 1.1% for StkK-3QV (\( n = 3 \)) (Fig. 7C). This large fractional activation by cGMP further supports the conclusion that cGMP is a partial agonist on StkK channels, not an antagonist.

The dramatic increase in fractional activation between these two channels reflects a large energetic change in the opening favorability produced by the A208V, R284Q, and E290Q mutations. To determine the magnitude of energetic change, we calculated \( L_{cGMP} \) for each channel. For cfStkK-L422Q, the 2.5% fractional activation by cGMP corresponds to \( L_{cGMP} \sim 0.025 \) (Fig. 7C). For cfStkK-3QV, making the conservative estimate that \( P_o = 0.92 \) in saturating cAMP, we calculated a \( P_o \) in cGMP
of ≈0.84, which corresponds to $L_{ccGMP} \approx 5.3$ (Fig. 7, B and C). This analysis suggests that the three mutations outside the CNBD introduced to generate the 3QV construct (i.e. not including L422Q) increased the liganded opening equilibrium constant, $L$, by at least 200-fold, corresponding to a $\Delta \Delta G$ of $-3.2$ kcal/mol. The cfSthK-3Q construct (i.e. lacking the
A208V mutation) exhibited an intermediate level of cGMP fractional activation, indicating that the C-linker mutations and the S6 mutation both contribute substantially to the increased opening favorability of cfSthK-3QV (Fig. 7C, right).

The large increase in $L_o$ was further apparent in the dose-response curve for cAMP in cfSthK-3QV. Although wtSthK produced a $K_{1/2}$ value of 1.5 μM, the $K_{1/2}$ of cfSthK-3QV was shifted by about 50-fold to a value of 32 ± 5 nM with $n = 2.2 ± 0.2$ ($n = 3$) (Fig. 7D). Furthermore, the large increase in $L_o$ for the cfSthK-3QV channel allowed a dose-response curve to be acquired for cGMP (Fig. 7D). The cGMP dose response can be fit with $K_{1/2} = 5.5 ± 0.3$ μM and $n = 1.9 ± 0.1$ ($n = 3$). Notably, the observation that mutations known to increase opening favorability in CNG channels also do so in SthK further suggests that SthK is mechanistically similar to CNG channels and is a viable model to study CNG gating (6, 19, 35).

The combination of higher cAMP apparent affinity, higher cGMP efficacy, and higher $P_o$ at hyperpolarized voltages made cfSthK-3QV expression highly toxic to $E$. coli. C43 cells transformed with cfSthK-3QV showed greatly diminished growth in culture, even compared with cfSthK (Fig. 7E). However, cultures of cfSthK-3QV in C43 cyA− cells still grew at a similar rate and reached a similar density as cultures expressing cfSthK (Fig. 7E). These results demonstrate that the strategy of eliminating cAMP from the E. coli cytoplasm is still sufficient to reduce the apparent toxicity of cfSthK-3QV expression.

**Discussion**

Prokaryotic channels have the potential to provide major insights into the gating mechanism of CNG channels. Here, we attempted to establish SthK as a model system for studying CNG gating.

First, we characterized the functional properties of wtSthK in E. coli membranes. SthK has been characterized previously using different systems, either expressed in Xenopus oocytes or reconstituted in artificial membranes (21, 22). The functional properties that we measured for SthK in bacterial spheroplasts exhibit a number of important differences from the properties reported previously. Most notably, the $P_o$, that we observed at saturating cAMP concentration (0.61 and 0.90 at −60 and +60 mV, respectively) was much higher than measured in oocytes (0.14 at +100 mV) or artificial membranes (0.65 at +200 mV) (21, 22). Assuming a simple closed–open equilibrium, our $P_o$ corresponds to nearly a 2 kcal/mol stabilization of the open state. Furthermore, the correspondence between the single-channel $P_o$ and the relative conductance at +60 and −60 mV suggests that $P_o$ approaches 1 at highly depolarized voltages (Fig. 4B). This more favorable opening transition is also reflected in a higher apparent affinity for cAMP (1.5 μM) compared with oocytes (3.7 μM) and artificial membranes (17 μM) (21, 22). These results indicate that wtSthK displays a much more favorable opening transition in bacterial membranes.

There are a number of possible explanations for the observed difference in $P_o$. Although our wtSthK construct contains the native SthK C-helix sequence, the construct used previously for reconstitution and structural experiments contained a C-helix truncation that resulted in the C-helix mutations E421L and L422E (8, 22). These residues directly interact with cAMP in the X-ray crystal structure of the SthK C-linker/CNBD fragment (see Fig. 6B) (29). Furthermore, the C-helix is thought to form an open state–dependent interaction with the cNMP (Fig. 6B). This interaction is thought to be largely responsible for the f-fold change in cAMP affinity that drives the opening transition, L (Fig. 1B). Therefore, disruption of this interaction could lower f and alter gating. Furthermore, several studies in CNG and HCN channels have shown that a negatively charged residue in the position equivalent to 422 substantially reduces cAMP efficacy (19, 38, and 45). The ~2 kcal/mol change in gating favorability is on the order of previously reported energetic interactions in this region (19). Consequently, it seems plausible that the differences in maximum $P_o$ could be due, in part, to differences in C-helix sequence. However, in oocytes, a low $P_o$ was reported for the full-length wtSthK construct, which suggests that other factors are also at work (21).

A second notable difference in our studies is that our recording solutions include 20 mM Mg2+ whereas neither of the other two studies included Mg2+ in their recording solutions (21, 22). Mg2+ was required to inhibit endogenous E. coli channels in the spheroplasts. Rapid Mg2+ block could potentially explain the lower single-channel conductance observed in our studies (Fig. 4E) compared with previous studies (22). However, it is unknown whether Mg2+ blocks SthK or whether the block is voltage-dependent and/or state-dependent. If Mg2+ block is open state–dependent, it would increase the apparent $P_o$ of the channel by mass action. The value for $L_o$ in the presence of Mg2+ would increase to $L_o(1 + M)$ with $L_o$ representing the closed–open equilibrium constant in the absence of block and M representing the open–block equilibrium constant (Fig. 1B). However, a ~26-fold increase in $L_o$ would require that $M$ be ~25. In which case, the single-channel current would be reduced to $1/26$ of that previously measured. Therefore, this mechanism alone could not account for an increase in $L_o$ of this magnitude.

A third possible explanation for the differences is that the bacterial membrane itself may enhance opening favorability. An 86Rb+ uptake assay showed that reconstitution of SthK in proteoliposomes containing 20% cardiolipin (CL) appeared to increase SthK activity (22). CL is a charged lipid that is composed of two phosphatidylglycerols linked by an additional glycerol molecule. The previous recordings of SthK were done.

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**Figure 7. Characterization of cfSthK-3QV construct.** A cartoon representation of SthK (PDB code 6CJQ in the middle and left panels and PDB code 4D7T on the right) showing locations of three Gin residues and one Val residue in the 3QV construct. B, representative single-channel recording of cfSthK-3QV in 1 mM cAMP. An all-points histogram is shown on the right. The blue line represents fit to a sum of Gaussians with $P_e = 0.92$ for this patch. C, left, leak-subtracted currents in cAMP (red traces) and cGMP (green traces) for cfSthK-62QV and cfSthK-3QV channels. Right, currents at −60 mV in cGMP relative to cAMP for cfSthK-L422Q, cfSthK-3Q, and cfSthK-3QV. Bars and error bars represent mean ± S.E., respectively, for three patches. D, dose-response curves for cfSthK-3QV in cAMP (red circles) and cGMP (green squares). The solid black line represents Hill fit with $K_{1/2} = 32$ nM and $n = 2.2$ and with $K_{1/2} = 5.5$ μM and $n = 1.9$ for cAMP and cGMP, respectively. The dashed black line represents cAMP dose response for wtSthK. Points and error bars represent mean ± S.E., respectively, for three patches. E, growth curves of C43 and C43 cyA− cells with cfSthK and cfSthK-3QV. Expression of the indicated SthK construct was induced between an OD600 of 0.4 and 0.6. Points and error bars represent mean ± S.D., respectively, for three cultures.
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in oocytes or bilayers that contained little or no CL. The *E. coli* inner membrane, however, contains about 5–10% CL (39, 40). CL is known to regulate bacterial inner-membrane proteins as well as mitochondrial inner-membrane proteins (41–43). Additionally, many ion channels are strongly regulated by charged lipids such as phosphatidylinositol phosphates (44). This suggests that the ~2 kcal/mol increase in opening favorability could indeed come, in part, from interaction with a charged lipid such as CL.

A feature of SthK on which previous studies disagree pertains to the effect of cGMP binding. In oocytes, no SthK current was elicited in the presence of cGMP, and cGMP inhibited activation of the channels by cAMP (21). These authors concluded that cGMP acts as a competitive inhibitor by binding to the CNBD but not promoting activation of the channel (*f* ≃ 1) (Fig. 1B). In artificial membranes, cGMP was also reported to inhibit cAMP-induced currents. However, these authors observed a significant single-channel *P* _o_ in the presence of cGMP alone. Therefore, they concluded that cGMP acts as a weak partial agonist on SthK (*f* is small but >1) (Fig. 1B) (22). Our results also indicate that cGMP acts as a partial agonist of SthK in bacterial membranes, but likely with a lower efficacy than previously reported. This difference in cGMP efficacy may result from the C-helix mutations in the previous study at positions known to affect the relative activation by cAMP and cGMP. Indeed, a Glu at the position equivalent to 422 has been shown to dramatically increase the cGMP activation of both mammalian CNG and HCN channels (19, 45).

The ideal biochemical model for structure–function studies of CNG channel gating would be a channel that is Cys-free, is nontoxic, and has a high *P* _o_ in the presence of ligand. We have produced such a model in the cfSthK-3QV construct. Removal of the two native Cys residues did not noticeably alter the gating properties (Fig. 5). Generation of the C43 *cyaA* ~ E. coli ~ strain eliminated the toxicity of SthK expression (Fig. 6). Finally, addition of mutations in SthK (cfSthK-3QV) substantially increased the *P* _o_ (Fig. 7). Importantly, the cfSthK-3QV construct could still be grown in the C43 *cyaA* ~ cells. This construct provides a foundation for future experiments of CNG channel gating.

**Experimental procedures**

**Bacterial spheroplasts**

The production of bacterial spheroplasts was done as described previously with some modification (24–26). SthK constructs (UniProt accession no. E0RR11) were cloned into a pCGFP vector (46). *E. coli* C43 cells were transformed with the indicated construct and streaked onto a 2× YT plate containing 100 μg/ml carbenicillin. A single colony was picked from a freshly transformed plate and inoculated into 10 ml of 2× YT medium containing 100 μg/ml carbenicillin. The culture was incubated at 37 °C with 220 rpm shaking until the OD _600_ reached ~0.3. 1 ml of this culture was then diluted into 10 ml of fresh 2× YT prewarmed at 42 °C and containing 100 μg/ml carbenicillin and 60 μg/ml cephalaxin (from a 10 mg/ml stock in H2O). The culture was incubated at 42 °C with 180 rpm shaking for 1.5 h. To make spheroplasts for single-channel recordings, the culture was then moved to 37 °C and shaken at 150 rpm for 20 min. Then 0.4 mM IPTG was added, and the culture was incubated for an additional ~20 min at 37 °C. To make spheroplasts for macroscopic recordings, the culture was removed from the 42 °C incubator and placed at 19 °C with 150 rpm shaking for 20 min. Then 0.4 mM IPTG was added, and the culture was incubated overnight at 19 °C with 150 rpm shaking. 1 ml of culture was removed and spun at 5000 rpm at 4 °C for 6 min. The supernatant was discarded. All of the following steps were performed at room temperature. The pellet was gently resuspended in 500 μl of 0.8 M sucrose. The following solutions were added in succession (inverting tube several times after each addition): 30 μl of 1 M Hepes, pH 7.4; 24 μl of 0.5 mg/ml lysozyme (freshly prepared in H2O); 6 μl of 5 mg/ml DNase I (freshly prepared in H2O); and 6 μl of 125 mM EDTA, pH 8. Spheroplast formation was monitored under the microscope (40× objective). After ~4–7 min, 100 ml of stop solution (0.7 M sucrose, 20 mM MgCl2, 10 mM Hepes, pH 7.4) was added, and the tube was inverted multiple times. The sample was aliquoted into 50-μl fractions and placed directly into a ~80 °C freezer for storage.

**Fluorescence imaging**

Spheroplast imaging was performed on a Nikon Eclipse TE2000-E microscope with a 60× water immersion objective (numerical aperture, 1.2). Images were acquired on an Evolve 512 EMCCD camera (Photometrics) using the program MetaMorph (Molecular Devices). The images were analyzed in ImageJ (National Institutes of Health).

**Electrophysiology**

Symmetrical solutions containing 150 mM KCl, 20 mM MgCl2, 500 mM sucrose, 10 mM Hepes, pH 7.4, were used both in the pipette and in the bath. Patch pipettes were pulled from borosilicate glass tubes without polishing to an open pipette resistance of 2–4 megaohms for macroscopic recordings and 5–8 megaohms for single-channel recordings. Spheroplasts were thawed from ~80 °C, and 12–20 μl was added to the bath and allowed to settle to the bottom for ~20 min. A gigaohm seal was formed on the membrane. Then the pipette was brought away from the bottom of the dish, and the head stage was flicked to excise the patch in an inside-out configuration. Spheroplasts were patched within 1 h after addition to the dish.

Data were acquired using an Axopatch 200A amplifier with Patchmaster software (HEKA Elektronik). For single-channel recordings, the data were sampled at 20 kHz and low-pass filtered at 2 kHz. For recordings at +60 mV, data were further filtered at 1 kHz. For macroscopic recordings, the data were sampled at 10 kHz and low-pass filtered at 2 kHz. Single-channel currents were recorded at a holding potential of ~60 mV unless otherwise indicated. Perfusion was achieved through an RSC-100 rapid change solution changer (Biologic). For dose-response curves, steady-state currents were measured at ~60 mV. Macroscopic currents recorded in the presence of cAMP and cGMP were leak-subtracted using identical voltage protocols in the absence of ligand to remove leak and capacitance currents.
Data analysis

Data were analyzed using Igor (Wavemetrics), QuB express (47), and Microsoft Excel. Dose-response curves were fit with the Hill equation,

\[ \frac{I}{I_{\text{max}}} = \frac{1}{1 + (K_{1/2}/[\text{cNMP}])^h} \]  

(Eq. 1)

where \( I_{\text{max}} \) represents the maximal current in saturating cAMP, \( K_{1/2} \) represents the concentration of cNMP producing half-maximal current, and \( h \) represents the Hill coefficient.

\( G-V \) curves were fit with the Boltzmann equation,

\[ \frac{I}{I_{\text{max}}} = \frac{1 - I_{\text{max}}/I_{\text{max}}}{1 + e^{z(\delta-V)/V_{1/2}}} \]  

(Eq. 2)

where \( I_{\text{max}} \) represents the maximal leak-subtracted current measured 2.5 ms after stepping to +100 mV, \( z\delta \) represents the equivalent charge movement, \( F \) represents Faraday’s constant, \( R \) represents the universal gas constant, and \( T \) represents absolute temperature.

Single-channel recordings were analyzed by the accumulation of all data points into a histogram. Histograms from patches containing a single channel were fit with a sum of two Gaussians.

\[ \text{Counts} = C[(P_o)e^{-0.5(x-i/\alpha)^2} + (1 - P_o)e^{-0.5(x+i/\alpha)^2}] \]  

(Eq. 3)

Currents from patches containing two or more channels were fit with polynomial distributions, Equation 4 for two channels and Equation 5 for three channels,

\[ \text{Counts} = C[(P_o)^2e^{-0.5(x-2i/\alpha)^2} + 2(P_o)(1-P_o)e^{-0.5(x-i/\alpha)^2} + (1-P_o)^2e^{-0.5(x+i/\alpha)^2}] \]  

(Eq. 4)

\[ \text{Counts} = C[(P_o)^3e^{-0.5(x-3i/\alpha)^2} + 3(P_o)^2(1-P_o)e^{-0.5(x-2i/\alpha)^2} + 3P_o(1-P_o)^2e^{-0.5(x-i/\alpha)^2} + (1-P_o)^3e^{-0.5(x+i/\alpha)^2}] \]  

(Eq. 5)

where \( C \) scales the amplitude of the Gaussians to the number of counts, \( i \) represents the single-channel current, \( \alpha \) represents the variance, and \( P_o \) represents the single-channel open probability.

The opening equilibrium constant, \( L \), was calculated using the following equation.

\[ L = P_o/(1-P_o) \]  

(Eq. 6)

The change in free energy of opening was calculated using the following equation,

\[ \Delta\Delta G = -RT \ln(L1/L2) \]  

(Eq. 7)

where \( R \) represents the universal gas constant, \( T \) represents the absolute temperature, and \( L1 \) and \( L2 \) represent equilibrium constants.

Generation of cyaA\(^{-}\) E. coli

Adenylate cyclase–deficient E. coli were generated by oligonucleotide-mediated recombination as described previously (Table 1) (32). C43(DE3) E. coli (Lucigen) were transformed with temperature-sensitive helper plasmid pSJ8, which encodes the ARed recombinase proteins Gam, Beta, and Exo under control of the araBAD promoter as well as a rhamnose-inducible flippase recombinase (FLP) (48). Transformed cells were grown in 2× YT medium at 30 °C with 100 μg/ml carbenicillin until reaching an OD\(_{600}\) of ∼0.4, at which point the ARed proteins were induced with 0.25% L-arabinose. Induced cells were grown for an additional 30 min before making electrocompetent by concentrating ∼100-fold and washing (four times) with 10% glycerol. Aliquots of ARed-induced electrocompetent cells were stored at −80 °C until use.

The chlomorphenicol resistance cassette, flanked by flippase recognition target sequences (FRT-cat-FRT), was PCR-amplified from plasmid pKD3 using KOD polymerase (Novagen) and primers containing 36–38-nucleotide extensions homologous to regions immediately flanking the cyaA gene locus of C43. The resulting ∼1.1-kbp PCR product was gel-purified.

3 μl (141 ng) of FRT-cat-FRT dsDNA was mixed with 50 μl of electrocompetent C43 cells expressing the ARed system and transferred to a chilled electroporation cuvette with a 1-mm gap. Cells were shocked using a Bio-Rad GenePulser (1.8 kV, 25 microfarads, 250 ohms) and recovered in 0.5 ml of 2× YT medium at 30 °C for 2 h. Cells were plated on LB agar containing 100 μg/ml carbenicillin and 10 μg/ml chloramphenicol and incubated at 30 °C. Colonies were visible after ∼3 days at 30 °C, and successful recombinants were inoculated into 4 ml of 2× YT with 100 μg/ml carbenicillin and 20 μg/ml chloramphenicol. After overnight growth at 30 °C, cultures were streaked onto plates containing 100 μg/ml carbenicillin and 20 μg/ml chloramphenicol and again grown at 30 °C. This cycle of colony purification was then repeated a second time. Loss of the cyaA gene and simultaneous gain of the FRT-cat-FRT cassette was confirmed by colony PCR using JumpStart Taq Ready Mix (2×) (Sigma) and locus-specific as well as insert-specific primer pairs. Finally, removal of the helper plasmid pSJ8 was achieved by overnight growth at 37 °C in 2× YT + 20 μg/ml chloramphenicol. Cells from the resulting strain, termed C43 cyaA\(^{-}\), were made electrocompetent and stored in aliquots at −80 °C.

Growth curves

For growth analysis, bacteria were grown in 10-ml cultures of 2× YT medium in 50-ml Mini Bioreactor tubes (Corning) with 220 rpm shaking. Optical densities were recorded at 600 nm from 10-fold dilutions of bacterial cultures using a Beckmann DU-800 spectrophotometer. Untransformed C43 and C43

| Table 1 | Strains, plasmids, and primers used in cyaA knockout |
|---------|-------------------------------------------------------|
| **Template Strain** | **Description** | **Reference** |
| C43(DE3) | Lucigen Corp. | F – emp/ lacI hsdS (rB- mB-) gal dcm (DE3) | (49) |
| **Plasmid** | **Addgene plasmid #** | **Reference** |
| pSJ8 | 68122 | (48) |
| pKD3 | 45604 | (32) |
| **Primer Sequence** | **Reference** |
| cyaA_FRTcatFRT_f | CACAGTCATGGACGGGTACGATACGTCTGTCGAACATTCGCAGCTACCTTA | (48) |
| cyaA_FRTcatFRT_r | CACAGTCATGGACGGGTACGATACGTCTGTCGAACATTCGCAGCTACCTTA | (48) |
| colPCR_Locus_f | TGTGATAGGCTGGAGCTGCCTTTTCTC | (32) |
| colPCR_Locus_r | CTGCCTTTTCTCCTGAG | (32) |
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cyA− cells in the absence of antibiotics were grown at 37 °C, whereas SthK-transformed bacteria were grown at 32 °C and included 50 μg/ml kanamycin. Bacteria were selected from single colonies and grown overnight to saturation. The following day, the cultures were diluted to OD600 < 0.05, induced with 0.5 mM IPTG when OD600 reached 0.45–0.65, and moved to 20 °C. Alternatively, SthK-transformed colonies were resuspended directly from plates and diluted to OD600 ≈ 0.4 in 2× YT medium containing 50 μg/ml kanamycin and 1 mM IPTG, and growth was monitored at 32 °C.

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