cAMP acts as a second messenger in many cellular processes. Three protein types mainly mediate cAMP-induced effects: PKA, exchange protein directly activated by cAMP (Epac), and cyclic nucleotide–modulated channels (cyclic nucleotide–gated or hyperpolarization-activated and cyclic nucleotide–modulated (HCN) channels). Discrimination among these cAMP signaling pathways requires specific targeting of only one protein. Previously, cAMP modifications at position N^6 of the adenine ring (PKA) and position 2'-OH of the ribose (Epac) have been used to produce target-selective compounds. However, cyclic nucleotide–modulated ion channels were usually outside of the scope of these previous studies. These channels are widely distributed, so possible channel cross-activation by PKA- or Epac-selective agonists warrants serious consideration. Here we demonstrate the agonistic effects of three PKA-selective cAMP derivatives, N^6-phenyladenosine-3',5'-cyclic monophosphate (N^6-Phe-cAMP), N^6-benzyladenosine-3',5'-cyclic monophosphate (N^6-Bn-cAMP), and N^6-benzoyl-adenosine-3',5'-cyclic monophosphate (N^6-Bnz-cAMP), on murine HCN2 pacemaker channels. Electrophysiological characterization in Xenopus oocytes revealed that these derivatives differ in apparent affinities depending on the modification type but that their efficacy and effects on HCN2 activation kinetics are similar to those of cAMP. Docking experiments suggested a pivotal role of Arg-635 at the entrance of the binding pocket in HCN2, either causing stabilizing cation–π interactions with the aromatic ring in N^6-Phe-cAMP or N^6-Bn-cAMP or a steric clash with the aromatic ring in N^6-Bnz-cAMP. A reduced apparent affinity of N^6-Phe-cAMP toward the variants R635A and R635E strengthened that notion. We conclude that some PKA activators also effectively activate HCN2 channels. Hence, when studying PKA-mediated cAMP signaling with cAMP derivatives in a native environment, activation of HCN channels should be considered.
HCN2 channel activation by N^6- modified cAMP derivatives

(17). This similarity in the cAMP-binding mechanism of all cAMP-responsive proteins makes it challenging to target only one type of protein to discriminate between the different cAMP signaling pathways. However, discrimination between two proteins, PKA and Epac, could be realized and has been used widely (e.g. (18–22)). Although Epac tolerates 2'-OH modifications, modifications at the N^6 position of the purine ring are not accepted by this protein. However, such N^6-modified derivatives are often potent agonists for PKA and can be used to exclude Epac activation (23). Notably, fully functional ion channels were often outside the scope when studying the selectivity of cAMP derivatives.

Besides a systematic study performed by Ng et al. (24) comparing binding and gating parameters for different cyclic nucleotides in whole HCN channels, the effect of purine ring modifications at cAMP on functional channels have so far not been tested systematically. To contribute to the urgent problem of developing cAMP analogs that can discriminate between the different cAMP-binding proteins, here we tested three N^6-modified cAMP derivatives: N^6-phenyladenosine-3',5'-cyclic monophosphate (N^6-Phe-cAMP), N^6-benzyladenosine-3',5'-cyclic monophosphate (N^6-Bn-cAMP), and N^6-benzoyladenosine-3',5'-cyclic monophosphate (N^6-Bnz-cAMP), known to be activators of PKA, on functional HCN2 channels. We show that all of them are HCN2 channel activators with efficacies similar to cAMP but with different apparent affinities. Docking and mutagenesis experiments revealed the specific interactions between molecule and binding pocket that underlie those differences.

Results

Effect of the native ligand cAMP on HCN2 channels in oocyte macropatches

HCN2 channels produce slowly activating inward currents in response to hyperpolarizing voltage jumps. It has been shown that the cyclic nucleotide-binding domain has an inhibitory effect on channel gating that is relieved by cAMP binding (13). Such a relieving effect is reflected by an increase in current amplitude at a given voltage, by a shift of the voltage of half-maximum activation, \( V_{1/2} \), to more depolarized values, and by an acceleration of activation kinetics and a deceleration of deactivation kinetics (14). This could also be observed here for mHCN2 channels expressed in Xenopus laevis oocytes (Fig. 1).

Fig. 1. A and B, shows representative current traces and the respective protocols used to determine channel activation at varying agonist concentrations at a given command voltage of -130 mV (Fig. 1A) and to determine channel activation at varying voltages at zero or saturating agonist concentrations (Fig. 1B), respectively. The cAMP concentration required to cause EC_{50}, a measure of the apparent affinity of the agonist to the receptor, was determined by approximating the Hill equation (Equation 1) to relative current amplitudes plotted against the cAMP concentration (Fig. 1C). Current amplitudes were obtained from tail currents at -100 mV following an activating pulse of -130 mV. The EC_{50} value was found to be 21.3 ± 3.3 nM, and the Hill coefficient was 1.1 ± 0.2. From this, 10 \u03bcM was defined as the saturating concentration for all experiments.

To estimate \( V_{1/2} \), relative current amplitudes were plotted versus the command voltage. The Boltzmann equation was fitted to the data points of individual recordings, yielding \( V_{1/2} \) under control conditions of -117.8 ± 1.5 mV and after application of 10 \u03bcM cAMP of -107.9 ± 1.9 mV. Thus, the steady-state activation was shifted by 19.9 ± 1.2 mV to more depolarized values because of cAMP binding (Fig. 1D). The slopes of

\[ \text{Figure 1. CAMP effects on steady-state and nonsteady state parameters of mHCN2 channel activation.} \]

A. protocol and representative current traces to study concentration-dependent gating at a fixed command voltage. Tail currents were obtained from a -100 mV pulse following an activating -130 mV pulse. B. protocol and representative current traces (example of a saturating concentration of 10 \u03bcM CAMP) to study voltage-dependent gating at a fixed agonist concentration. A voltage family from -70 mV to -150 mV was applied with 10-mV increments. Tail currents were obtained from a -100 mV pulse following the variable test pulse. C. concentration–response relationship for cAMP. Mean values for \( I/I_{max} \) were obtained from six to 13 recordings and plotted against the cAMP concentration. The Hill equation (Equation 1) was approximated to the data, yielding \( E_{C_{50}} (21.3 ± 3.3 \text{ nM}) \) and \( H (1.1 ± 0.2) \), respectively. D. steady-state activation relationship at zero and saturating [cAMP]. Mean values for relative current amplitudes (\( I/I_{max} \)) for zero cAMP (n = 14) and for saturating cAMP of 10 \u03bcM (n = 13) were plotted against the command voltage. The Boltzmann equation was approximated to the data, yielding \( E_{C_{50}} (-117.8 ± 1.5 \text{ mV}) \) and a slope of \( z = 4.2 ± 0.3 \) for zero and \( V_{1/2} \) for 10 \u03bcM cAMP. E. activation kinetics at different cAMP concentrations. Activity time constants were obtained from approximating a monoexponential function (Equation 3) to the current time courses, yielding \( \tau_{a,conc} \). Mean values were obtained from three to seven recordings. The protocol used is shown in A. F. activation kinetics at different command voltages at zero and saturating [cAMP]. Activation time constants were obtained from approximating a monoexponential function (Equation 3) to the current time courses, yielding \( \tau_{a,voltage} \). Mean values were obtained from 14 recordings for zero and 13 recordings for 10 \u03bcM cAMP. The protocol used is shown in B.
the curves were similar with and without cAMP (4.2 ± 0.2 and 4.2 ± 0.3 mV/e-fold change, respectively).

The time constant of activation, given as \( \tau_{a,\text{conc}} \), decreased with increasing cAMP concentrations (Fig. 1E). Furthermore, the time constant of activation decreased with increasing hyperpolarizing voltages under control conditions without cAMP as well as after application of 10 μM cAMP (Fig. 1F).

**All three tested cAMP derivatives were able to promote HCN2 channel activation**

We tested three cAMP derivatives, used as PKA activators, for their ability to activate HCN2 channels: \( N^6 \)-Phe-cAMP, \( N^6 \)-Bn-cAMP, and \( N^6 \)-Bnz-cAMP (Fig. 2A). In these derivatives, a phenyl group, a benzyl group, or a benzoyl group, respectively, is attached to the amino group in position 6 of the adenine moiety. All three \( N^6 \)-modified derivatives have been shown to be site-selective for site A of both PKA type I and II (27–29).

To test whether the three cAMP derivatives are able to modulate HCN2 channels, channel activation was monitored at zero cAMP and in the presence of different agonist concentrations. Representative current recordings for zero cAMP and saturation are shown in Fig. 2B. All three derivatives caused an increase in current amplitude and acceleration of the activation kinetics, as known for cAMP (Fig. 1).

**cAMP modifications at position \( N^6 \) affected the apparent affinity of the agonist for HCN2**

To study the concentration dependence in more detail, concentration–response relationships were recorded for each derivative (Fig. 2C) and compared with the relationship for cAMP. For all cases, the Hill equation (Equation 1) was approximated to the relative currents of each individual recording. The results for \( EC_{50} \) and the Hill coefficient, \( H \), are summarized in Fig. 2, D and E, respectively. The nature of the \( N^6 \) modification had a major influence on the apparent affinity (\( EC_{50,\text{Phe}} = 3.02 \pm 0.83 \, \text{nM} \), \( EC_{50,\text{Bn}} = 5.12 \pm 1.03 \, \text{nM} \), \( EC_{50,\text{Bnz}} = 374 \pm 91.3 \, \text{nM} \) (Fig. 2D). Adding a phenyl or benzyl group to position \( N^6 \) shifted the \( EC_{50} \) value to lower concentrations compared with native cAMP, causing an apparent affinity one order of magnitude higher than for native cAMP.
cAMP. However, adding a benzoyl group shifted the EC$_{50}$ value to higher concentrations, indicating an apparent affinity one order of magnitude lower than for cAMP (Fig. 2). The Hill coefficients for the derivatives were not significantly different from that for cAMP: $H_{\text{Phe}} = 1.4 \pm 0.3$, $H_{\text{Bn}} = 1.2 \pm 0.3$, and $H_{\text{Bnz}} = 0.9 \pm 0.2$ (Student’s $t$ test, $p = 0.05$) (Fig. 2E).

None of the cAMP modifications tested changed the efficacy

We used two measures to compare the efficacy of the cAMP derivatives with that of native cAMP: the maximal agonist-induced voltage shift ($\Delta V_{1/2,\text{max}}$) and the maximal fractional increase in tail current amplitude ($I_{\text{max,agonist}}/I_{\text{max,cAMP}}$) observed with saturating agonist concentrations (Fig. 3) (30).

To yield $\Delta V_{1/2,\text{max}}$, $V_{1/2}$ values before and after application of saturating agonist concentrations were estimated by fitting the Boltzmann equation to steady-state activation relationships (Fig. 3A). There was no difference between $\Delta V_{1/2,\text{max}}$ of cAMP and either one of the tested derivatives (16.1 ± 2.3 mV for cAMP, 18.3 ± 1.4 mV for $N^6$-Phe-cAMP, 18.6 ± 2.4 mV for $N^6$-Bn-cAMP, and 22.3 ± 1.1 mV for $N^6$-Bnz-cAMP) (Fig. 3B).

To yield the maximal fractional increase in tail current amplitude, we determined the tail current amplitudes at a test pulse of $-100$ mV, following a hyperpolarizing pulse of $-130$ mV, for each cAMP derivative at saturating concentrations and related that to the maximum tail current amplitude of 10 $\mu$M cAMP in the same patch. All derivatives caused a fractional current of around 1.0, suggesting that they all cause a similar current increase as cAMP (Fig. 3C). The values were $1.02 \pm 0.14$ for $N^6$-Phe-cAMP, $0.98 \pm 0.05$ for $N^6$-Bn-cAMP, and $1.00 \pm 0.02$ for $N^6$-Bnz-cAMP (Fig. 3B). Thus, both measures led us conclude that the modifications performed in the tested cAMP derivatives did not affect the efficacy of the agonists.

Non of the cAMP modifications tested changed activation kinetics

Fig. 4 summarizes the results regarding activation kinetics upon hyperpolarizing voltage jumps. First, we studied the concentration dependence of these kinetics. For this purpose, we applied the agonists at different concentrations, covering a wide range, and recorded the currents at a nearly saturating voltage of $-130$ mV. Kinetics were quantified by $\tau_{\text{conc}}$ obtained from approximating a monoexponential equation (Equation 3) to the current time courses. The values were plotted against the normalized concentration, which is the ratio of applied concentration and apparent affinity [agonist]/EC$_{50}$ (Fig. 4A). Such normalization was required because large differences in the EC$_{50}$ values for the derivatives and cAMP did not allow comparison at absolute concentrations. As a result, the concentration dependence of $\tau_{\text{conc}}$ for derivatives could be superimposed with that of cAMP.

Second, we studied the voltage dependence of activation kinetics. For this purpose, we applied a family of hyperpolarizing voltages at saturating agonist concentrations and in the absence of agonists, respectively. The results are shown in Fig. 4B. Analogous to the concentration dependence, the voltage dependence of $\tau_{\text{voltage}}$ could be superimposed with that of

Figure 3. Efficacy of the three tested derivatives. A, steady-state activation relationships at zero and saturating agonist concentrations. The graphs show the relationships of the tested derivatives at saturating concentrations and in the absence of any ligand. Except for $N^6$-Bnz-cAMP, for which 100 $\mu$M was used as a saturating concentration, the concentration was 10 $\mu$M for saturation. $I/I_{\text{max}}$ are mean values obtained from three to six recordings. The Boltzmann equation was approximated to the relative current values to yield $V_{1/2}$. The results of the fits were as follows: $-97.5 \pm 2.0$ mV for $N^6$-Phe-cAMP, $-104.9 \pm 2.7$ mV for $N^6$-Bn-cAMP, and $-98.3 \pm 1.4$ mV for $N^6$-Bnz-cAMP. The slopes were $3.4 \pm 0.2, 4.1 \pm 0.2$, and $4.4 \pm 0.3$, respectively. B and C, comparison of the efficiency of the three derivatives compared with cAMP in two measures. B, box plots of $I_{\text{max,agonist}}/I_{\text{max,cAMP}}$. Filled circles indicate individual recordings. Error bars indicate S.D. C, box plots of $\Delta V_{1/2}$. Filled circles indicate individual recordings. Error bars indicate S.D.

Figure 4. Activation kinetics for the tested cAMP derivatives. A, activation kinetics in dependence on agonist concentration at a given command voltage of $-130$ mV. $\tau_{\text{conc}}$ values, obtained from approximating a monoexponential equation (Equation 3) to the current time courses, are plotted against the normalized concentration, which is the ratio of [agonist]/EC$_{50}$. Black symbols in each plot illustrate the case for cAMP. The protocol used is shown in Fig. 1A. B, activation kinetics in dependence on command voltage at a saturating agonist concentration. $\tau_{\text{voltage}}$ values were plotted against the command voltage. Open symbols represent recordings in the absence of ligands and filled symbols in the presence of saturating agonist concentrations. Mean values were obtained from three to 19 recordings. The protocol used is shown in Fig. 1B.
cAMP. From these results, we conclude that all derivatives had a similar accelerating effect as the native ligand.

Molecular docking suggests a pivotal role of Arg-635 for determining the binding behavior of N\(^6\)-modified derivatives

To determine the structural basis of the higher apparent affinities of N\(^6\)-Phe-cAMP and N\(^6\)-Bn-cAMP and the lower apparent affinity of N\(^6\)-Bnz-cAMP, we predicted and compared the binding modes of cAMP, N\(^6\)-Phe-cAMP, N\(^6\)-Bn-cAMP, and N\(^6\)-Bnz-cAMP in mHCN2J by molecular docking (Fig. 5). The selected docking protocol was able to reproduce the crystallographically determined binding mode of cAMP with a heavy-atom RMSD of 0.60 Å (Fig. 5\(\text{A} - \text{D}\)), indicating that the binding modes of the N\(^6\)-substituted derivatives can be reliably predicted. The predicted binding modes of the cAMP moiety of N\(^6\)-Phe-cAMP and N\(^6\)-Bn-cAMP deviate only marginally from the native binding mode of cAMP (heavy-atom RMSDs of 0.73 Å and 0.91 Å, respectively; Fig. 5, B and C). Docking scores (XP GScores) for all three compounds were in a similar range (cAMP, ~13.53 kcal mol\(^{-1}\); N\(^6\)-Phe-cAMP, ~10.20 kcal mol\(^{-1}\); N\(^6\)-Bn-cAMP, ~11.54 kcal mol\(^{-1}\)).

Compared with cAMP, the predicted binding modes of N\(^6\)-Phe-cAMP and N\(^6\)-Bn-cAMP reveal stabilizing cation–π and/or π–π stacking interactions between the additional phenyl ring and the side chain of Arg-635 (Fig. 5, A–C), which can explain the higher apparent affinities of these two derivatives. This interaction may be more stable in the case of N\(^6\)-Phe-cAMP because of the more restricted conformational freedom of the N\(^6\)-ring bonds compared with the less restricted N\(^6\)-benzyl carbon bond in N\(^6\)-Bn-cAMP, which

Figure 5. Predicted binding modes of the tested cAMP derivatives. A–D, predicted binding modes of cAMP (A, gray; crystallographic pose (PDB code 1Q5O (25) shown in gold), N\(^6\)-Phe-cAMP (B, blue), N\(^6\)-Bn-cAMP (C, orange), and N\(^6\)-Bnz-cAMP (D, magenta). In A–C, hydrogen bonds to and from protein side chains are depicted as dashed yellow lines, hydrogen bonds to and from the protein main chain as dashed green lines, and cation–π interactions as dashed blue lines. E, predicted binding mode of the cAMP fragment in N\(^6\)-Bn-cAMP extended with a benzoyl group as found in N\(^6\)-Bnz-cAMP. The steric clash with Arg-635 resulting from this extension is highlighted (red box), explaining the inverted binding mode of N\(^6\)-Bnz-cAMP shown in D.

HCN2 channel activation by N\(^6\)-modified cAMP derivatives

At Rsch Cntr Julich Res library on January 27, 2020http://www.jbc.org/ Downloaded from
can explain the higher apparent affinity of \( N^6 \)-Phe-cAMP compared with \( N^6 \)-Bn-cAMP.

In contrast to the \( N^6 \)-Phe and \( N^6 \)-Bn derivatives, the orientation of the cAMP moiety in the predicted binding mode of \( N^6 \)-Bnz-cAMP did not match the crystallographic pose (RMSD of 8.66 Å) but was instead inverted (Fig. 5D) and associated with a markedly lower docking score (−6.11 kcal mol\(^{-1}\)). To determine why \( N^6 \)-Bnz-cAMP cannot adopt the binding mode favorable for cAMP and the other \( N^6 \)-substituted derivatives, we used the predicted binding mode of \( N^6 \)-Bn-cAMP as a template and replaced the benzyl moiety with a benzoyl moiety. Because of the planarity of the amide group in \( N^6 \)-Bnz-cAMP, the phenyl ring is then forced into an orientation in which it inevitably clashes with Arg-635 (Fig. 5E), resulting in a strongly disfavorable interaction.

**Substituting Arg-635 at the entrance of the binding pocket prevented the higher apparent affinity of \( N^6 \)-Phe-cAMP**

The arginine at position 635 was substituted with either alanine for neutralization (R635A) or glutamate for charge reversal (R635E). If Arg-635 is indeed involved in the higher apparent affinity of \( N^6 \)-Phe-cAMP and \( N^6 \)-Bn-cAMP, then the mutations should cause an apparent affinity that resembles that of cAMP to WT channels. To test this, the concentration-activation relationship was tested for \( N^6 \)-Phe-cAMP in both R635A and R635E. Both constructs, R635A and R635E, formed functional channels and expressed in high densities in *Xenopus* oocytes. First, we tested whether those constructs are still reactive to 10 \( \mu \)M cAMP. For WT channels, we found a substantial current increase and an acceleration of the activation speed (Fig. 6A). When studying the concentration-response relationships for \( N^6 \)-Phe-cAMP, we found, as expected, that the \( EC_{50} \) values for both constructs were increased (Fig. 6, B and C), 27.7 ± 5.5 \( \mu \)M for R635A and 33.9 ± 5.3 \( \mu \)M for R635E. They are not different from the \( EC_{50} \) values found for cAMP (21.3 ± 3.3 \( \mu \)M) in WT channels.

**Discussion**

**PKA agonists activate HCN2 channels**

Here we performed a comparative study of three cAMP derivatives, \( N^6 \)-Phe-cAMP, \( N^6 \)-Bn-cAMP, and \( N^6 \)-Bnz-cAMP, earlier described as activators of PKA, to investigate their effects on HCN2 channels. In the last decades, these derivatives have been shown to be useful tools when discrimination between activation of PKA and Epac was required (18–22). Interestingly, all three tested derivatives turned out to also be activators for HCN2 channels heterologously expressed in *Xenopus laevis* oocytes by promoting HCN2 channel gating in the presence of a primary hyperpolarizing voltage stimulus.

To study the agonistic effect in more detail, we determined the concentration–activation relationship and quantified \( EC_{50} \) as a measure of the apparent affinity and the Hill coefficient of activation. The apparent affinity was considerably affected by the type of \( N^6 \) modification: Substituting one of the \( N^6 \)-bound hydrogens with a phenyl or benzyl group dramatically increased the apparent affinity (seven times and four times higher than cAMP, respectively), whereas a benzoyl ring decreased it (18 times lower than cAMP). However, because \( EC_{50} \) is a function of both binding affinity and efficacy, this parameter alone does not allow any interpretation of the mechanisms behind the agonist-dependent differences. The Hill coefficients for all agonists were similar to the Hill coefficient for cAMP. Even though this parameter is often used to describe the magnitude of cooperativity in ligand binding to allosteric systems, we hesitate to interpret those numbers in detail because, when derived from electrophysiological measurements, the Hill coefficient is just an empirical description without physical meaning because it depends on both binding and gating (31).

Figure 6. Response to \( N^6 \)-Phe-cAMP after charge neutralization or reversal at position Arg-635 in the CNBD. A, representative current traces for R635A and R635E at zero and saturating cAMP. B, concentration–activation relationships for mHCN2, R635A, and R635E. The Hill equation (Equation 1) was approximated to the relative currents to obtain \( EC_{50} \) and the Hill coefficient. C, box plot of \( EC_{50} \) values. Filled circles indicate individual recordings. Error bars indicate S.D. \( EC_{50} \) values obtained with \( N^6 \)-Phe-cAMP for R635A and R635E, respectively, are not different from the \( EC_{50} \) value obtained for cAMP and mHCN2 (Student’s t test).
channels (33), those data further suggest consideration of all three \(N^6\)-modified derivatives also as full agonists. In addition to those steady-state parameters, we quantified the activation kinetics of the macroscopic current time course following a hyperpolarizing voltage jump. The kinetics of a macroscopic current reflect the kinetics of the underlying channels and are determined by the rate at which a new equilibrium occurs after a perturbation. We compared the effect of the \(N^6\)-modified derivatives under two conditions: at varying voltages in the presence of a saturating agonist concentration and at varying concentrations at a saturating voltage. For both conditions, the activation time constants for each of the three derivatives were not different from the activation time constants for cAMP. This supports the interpretation of efficacy that the transduction events that follow the initial binding reaction are similar for all tested agonists. Together, those data led us to suggest that the \(N^6\)-modified derivatives tested here are differentially potent in HCN2 channels because they differ in their ability to bind to the HCN2 binding sites.

Arginine 635 in the CNBD affects apparent affinities

We performed docking experiments to identify molecular interactions between \(N^6\) derivatives and binding site residues that might underlie the observed differences in apparent affinities. The binding modes of \(N^6\)-Phe-cAMP and \(N^6\)-Bn-cAMP are stabilized by a cation–π and/or π–π stacking interaction between the additional phenyl moiety and the side chain of Arg-635. The higher apparent affinity of \(N^6\)-Phe-cAMP compared with \(N^6\)-Bn-cAMP is likely explained by the more restricted conformational freedom of the amino-phenyl side chain in the former case, which leads to a reduced loss in configurational entropy upon binding. In contrast, the benzoyl moiety in \(N^6\)-Bnz-cAMP enforces coplanarity of the two aryl systems, which would lead to a clash between the phenyl ring and Arg-635 if the cAMP moiety of \(N^6\)-Bnz-cAMP adopted the usual binding mode in the cAMP binding site. Based on these results, we hypothesized that the cation–π and/or π–π stacking interaction between the phenyl ring and Arg-635 is the main cause of the differential apparent affinities of \(N^6\)-Phe-cAMP and \(N^6\)-Bn-cAMP versus \(N^6\)-Bnz-cAMP.

To test this hypothesis, we either neutralized or reversed the charge at position 635 by replacing the arginine with either alanine (R635A) or glutamate (R635E). Neutralization resulted in an apparent affinity of \(N^6\)-Phe-cAMP similar to the apparent affinity of cAMP for mHCN2 WT channels. From this result, we suggest that now \(N^6\)-Phe-cAMP interacts with R635A in a similar manner as cAMP with the mHCN2 WT. Reversing the charge by replacing the arginine with a glutamate led to an apparent affinity of \(N^6\)-Phe-cAMP slightly lower than that of cAMP for mHCN2 WT channels. This might be caused by a repulsive force between the aromatic ring of \(N^6\)-Phe-cAMP and the negative side chain of the newly introduced glutamate. Together, these data support the hypothesis resulting from the docking experiments, proposing a stabilizing or destabilizing interaction between \(N^6\) modifications and Arg-635. A role of Arg-635 for binding and selectivity was discussed earlier by Zhou and Siegelbaum (30). They described this residue as being involved in discriminating between cAMP and cGMP without contributing to efficacy.

Usability of \(N^6\) modifications in cAMP to discriminate between PKA and HCN channels

What can we learn from the results presented here regarding discrimination between PKA and HCN channel activation? To compare the apparent affinities of the derivatives at HCN2 channels with that at PKA, we plotted relative EC\(_{50}\) values for PKAI and PKAII obtained from the literature (EC\(_{50,\text{cAMP}}\) versus relative EC\(_{50}\) values for HCN2 obtained here (Fig. 7) (29, 34–36).

The very high apparent affinity of \(N^6\)-Phe-cAMP as well as the very low one of \(N^6\)-Bnz-cAMP for HCN2 channels brings the \(N^6\) position into focus for the search for discriminating ago-
HCN2 channel activation by N\textsuperscript{6}-modified cAMP derivatives

In summary, our analysis shows that possible cross-activation of CN-modulated ion channels should be taken into account when CN-dependent cellular processes are investigated with N\textsuperscript{6}-modified derivatives. To overcome this problem, we further suggest that, even with a missing all-or-nothing principle in protein activation by cyclic nucleotides, carefully performed concentration–activation relationships can be used to identify a concentration range in which selective protein activation is possible.

Experimental procedures

X. laevis oocytes as a heterologous expression system

Surgical removal of oocytes was performed under anesthesia (0.3% triamine, MS-222, Pharmaq Ltd., Fordingbridge, UK) from adult females of the South African claw frog X. laevis. The oocytes were treated with collagenase A (3 mg/ml, Roche) for 105 min in Ca\textsuperscript{2+}-free Barth’s solution containing 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl\textsubscript{2}, and 5 mM Heps (pH 7.5). After this procedure, oocytes of stages IV and V were manually dissected and injected with cRNA encoding either mHCN2 channels of Mus musculus (NM_008226) or the mHCN2 mutants R635A and R635E, respectively. After injection with cRNA, the oocytes were cultured at 18 °C for 2–6 days in Barth’s solution containing 84 mM NaCl, 1 mM KCl, 2.4 mM NaHCO\textsubscript{3}, 0.82 mM MgSO\textsubscript{4}, 0.41 mM CaCl\textsubscript{2}, 0.33 mM Ca(NO\textsubscript{3})\textsubscript{2}, and 7.5 mM Tris (pH 7.4). The procedures had approval from the authorized animal ethical committee of the Friedrich Schiller University Jena. The methods were carried out in accordance with the approved guidelines. Oocytes harvested in our own laboratory were complemented with ready-to-use oocytes purchased from Ecocyte Bioscience (Dortmund, Germany).

Molecular biology

The mouse HCN2 (accession number NM_008226) channel and the modified subunit variants were subcloned in front of the T7 promoter of pGEM-HCN2. The point mutations R635E and R635A were introduced by overlapping PCR using the following primers: PCR 1 forward, 5’-TCATACTCAGCGCGGCGCAGGTCTTC-3’ (restriction site XbaI, outer primer); PCR 1 reverse, 5’–CTCTTGGCTATCTCTAGCGGTCAATAGC-3’ (R635E) or 5’–CTCTTGGCTATCTCTAGCGGTCAATAGC-3’ (R635E); PCR 2 forward, 5’–GCTATGTGCGAGATGGAGGAGAAG-3’ (R635E) or 5’–GCTATGTGCGAGATGGAGGAGAAG-3’ (R635E); PCR 2 reverse, 5’–AGCAGGGTTTGTCCAGATCGATGGAGAAG-3’ (restriction site XbaI, outer primer). In a third PCR, the PCR products of PCR 1 and 2 were used as a template using the outer primers. The resulting fragment was subcloned into the pGEM-HCN2 subunit. Correctness of the sequences was confirmed by restriction analysis and sequencing (Microsynth SEQLAB). cRNAs were prepared using the mMESSAGE mMACHINE T7 Kit (Ambion).

Electrophysiological experiments

Macroscopic currents were recorded using the patch clamp technique in the inside-out configuration. All measurements were started after a delay of 3.5 min to minimize rundown phen-
HCN2 channel activation by N⁶-modified cAMP derivatives

nomina. Patch pipettes were pulled from quartz tubing whose outer and inner diameters were 1.0 and 0.7 mm (Vitrocom), respectively, using a laser puller (P-2000, Sutter Instrument). The pipette resistance was 1.2–2.1 megaohm. The bath solution contained 100 mM KCl, 10 mM EGTA, and 10 mM Hepes (pH 7.2), and the pipette solution contained 120 mM KCl, 10 mM Hepes, and 1.0 mM CaCl₂ (pH 7.2). For parts of the experiments, different concentrations of cAMP, N⁶-Phe-cAMP, N⁶-Bn-cAMP, or N⁶-Bnz-cAMP (BIOLOG LSI, Bremen, Germany) were applied with the bath solution. A saturating concentration of 10 μM cAMP was applied to each patch to define the maximum current amplitude. An HEKA EPC 10 USB amplifier (Harvard Apparatus) was used for current recording. Pulsing and data recording were controlled by the Patchmaster software (Harvard Apparatus). The sampling rate was 5 kHz. The holding potential was generally −30 mV.

Quantification and statistical analysis

Concentration–activation relationships were analyzed by approximating the Hill equation to each individual recording using the OriginPro 9.0G software.

\[ \frac{I}{I_{\text{max}}} = \frac{1}{[1 + (EC_{50}/[\text{agonist}])^n]} \]  

(\text{Eq. 1})

\( I \) is the actual current amplitude at a given agonist concentration, \( I_{\text{max}} \) the maximal current amplitude at a saturating concentration of 10 μM cAMP, EC_{50} the concentration of half-maximum activation, and \( H \) the Hill coefficient. Current amplitudes were generally obtained from tail currents at a −100 mV pulse following an activating −130 mV pulse and corrected for leak currents obtained from a short −100 mV pulse at the beginning of each trace. Values for EC_{50} and \( H \) were yielded for each individual recording and averaged. Steady-state activation curves were analyzed by fitting the Boltzmann equation to each individual recording using the OriginPro 9.0G software.

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

(\text{Eq. 2})

\( V_{1/2} \) is the voltage of half-maximum activation and \( z \delta \) the effective gating charge. \( F, R, \) and \( T \) are the Faraday constant, the molar gas constant, and the temperature in Kelvin, respectively. \( I \) is the actual current amplitude and \( I_{\text{max}} \) the maximum current amplitude at the saturating hyperpolarizing voltage of −150 mV specified for each patch.

The time courses of current activation were fitted with a single exponential starting after an initial delay.

\[ I(t) = A \times \exp\left[-t/\tau\right] \]  

(\text{Eq. 3})

\( A \) is the amplitude, \( t \) the time, and \( \tau \) the time constant for activation.

Experimental data are given as mean ± S.E. Statistical analysis was performed by an unpaired Student’s \( t \) test. A value of \( p < 0.05 \) was accepted as statistically significant.

Molecular modeling and docking experiments

Three-dimensional structures of the anionic species of cAMP, N⁶-Phe-cAMP, N⁶-Bn-cAMP, and N⁶-Bnz-cAMP were generated in Maestro (Schrödinger, LLC) and prepared using the LigPrep workflow (Schrödinger, LLC). One monomer (chain A) of the crystal structure of cAMP-bound mHCN2 (25) was prepared using the Protein Preparation Wizard in Maestro; the program was evoked to cap the termini with acetyl and N-methyl amide moieties and to convert selenomethionine residues back to methionine residues. The potential grid for docking was centered on the cocrystallized cAMP, and the ligand length was set to 16 Å or less. The dimensions of the inner box that restricts the region in which the diameter midpoint of each ligand can be located were set to 10 × 10 × 10 Å. No constraints, rotatable OH- and SH-groups, or excluded volumes were defined. Molecular docking was performed with Glide (Schrödinger, LLC) using the Extra Precision (26) mode with default options.


Acknowledgments—We thank Sandra Bernhardt, Uta Enke, Andrea Kolchmeier, Claudia Ranke, and Karin Schoknecht for excellent technical assistance. We are grateful for computational support and infrastructure provided by the Zentrum für Informations- und Medientechnologie at the Heinrich Heine University Düsseldorf and the computing time provided by the John von Neumann Institute for Computing (to H. G.) on the supercomputer JULWELS at the Jülich Supercomputing Center (user IDs HKF7 and HDD17).

References


N\textsuperscript{6}-modified cAMP derivatives that activate protein kinase A also act as full agonists of murine HCN2 channels

Tim Leypold, Michele Bonus, Felix Spiegelhalter, Frank Schwede, Tina Schwabe, Holger Gohlke and Jana Kusch

doi: 10.1074/jbc.RA119.010246 originally published online October 15, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA119.010246

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 41 references, 11 of which can be accessed free at http://www.jbc.org/content/294/47/17978.full.html#ref-list-1