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

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Running Title: HCN2 channel activation by N^6-modified cAMP derivatives

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ABSTRACT

cAMP acts as a second messenger in many cellular processes. Three protein types mainly mediate cAMP-induced effects: protein kinase A (PKA), exchange protein directly activated by cAMP (Epac), and CN-modulated channels (CNG or 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, CN-modulated ion channels were usually outside 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. Herein, 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-benzoyleadenosine-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 N⁶-Phe-cAMP or N⁶-Bn-cAMP, or a steric clash with the aromatic ring in N⁶-Bnz-cAMP. A reduced apparent affinity of N⁶-Phe-cAMP towards 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.

INTRODUCTION

Cyclic adenosine 3',5'-monophosphate (cAMP), the first identified second messenger (1), plays a key role in living organisms ranging from Dictyostelium to Homo sapiens. It is involved in a wide variety of cellular processes including proliferation, differentiation, secretion, migration, pacemaking, sensation, and apoptosis. The concentration of cytosolic cAMP is controlled by the action of adenyl cyclases, catalysing the conversion of ATP to cAMP, and cyclic nucleotide phosphodiesterases (PDE), catalysing the conversion of cAMP to 5'-AMP. In mammalian cells, four main types of proteins respond to cAMP: protein kinase A (PKA), exchange protein directly activated by cAMP (Epac1 and Epac2), CN-modulated ion channels (cyclic-nucleotide gated (CNG) channels and hyperpolarization-activated and cyclic-nucleotide modulated (HCN) channels) (2) and the relatively new class of popeye domain containing proteins (POPDC) (3). HCN channels, as one type of CN-modulated ion channels, are activated by hyperpolarizing membrane potentials as a primary stimulus but are secondarily stimulated by the direct binding of cyclic nucleotides, mainly cAMP, to intracellular binding sites (4-10). Structurally, HCN channels belong to the superfamily of tetrameric voltage-gated ion channels (11). In these channels each subunit contains a voltage-sensor domain and a pore domain. In contrast to most other members of this superfamily, HCN channel subunits contain additionally a cyclic nucleotide-binding domain (CNBD) in the C-terminus, which is connected to the membrane portion by a so-called C-linker (12). It has been shown that the unoccupied CNBD together with the C-linker has an inhibitory effect on channel gating, which is relieved by CN binding (13). Such a relieving effect is reflected by a shift of the steady-state activation curve to more depolarized voltages, thereby increasing the maximum current amplitude, accelerating the activation and decelerating the deactivation (14,15).

The cyclic-nucleotide binding domain (CNBD) is a highly conserved structure (16). Combined data from PKA, Epac and ion channel studies illustrate that CNBDs use a common mechanism to bind and sense cAMP (17). This similarity in the cAMP binding mechanisms for all cAMP-responsive proteins makes it challenging to target only one type of protein for discriminating between the different cAMP signalling pathways. However, discrimination between two proteins, PKA and Epac, could be realized and has been widely used (e.g. (18-22). While Epac tolerates 2'-OH-modifications, modifications at the N⁶-position of the purine ring are not accepted by this protein. However, such N⁶-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 selectivity of cAMP derivatives.

Besides a systematic study performed by Ng and co-workers comparing binding and gating parameters for different cyclic nucleotides in whole HCN channels (24), the effect of purine ring modifications at
cAMP on functional channels have not been tested systematically so far. To contribute to the urgent problem of developing cAMP-analogue which can discriminate between the different cAMP-binding proteins, we tested herein three N6-modified cAMP-derivatives: N6-Phenyladenosine-3',5'-cyclic monophosphate (N6-Phe-cAMP), N6-Benzyladenosine-3',5'-cyclic monophosphate (N6-Bn-cAMP), and N6-Benzoyladenosine-3',5'-cyclic monophosphate (N6-Bnz-cAMP), known to be activators for 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 exerts an inhibitory effect on channel gating, which 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, by an acceleration of activation kinetics and a deceleration of deactivation kinetics (14). This could also be observed herein for mHCN2 channels expressed in Xenopus laevis oocytes (Figure 1).

Figure 1 A and B show representative current traces and the respective protocols used to determine channel activation at varying agonist concentrations at a given command voltage of -130 mV (Figure 1A) and to determine channel activation at varying voltages at zero or saturating agonist concentrations (Figure 1B), respectively. The cAMP concentration required to cause half-maximum activation, \( EC_{50} \), a measure for 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 (Figure 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, the Hill coefficient was 1.1±0.2. From this, 10 µM was defined as saturating concentration throughout all experiments.

To estimate the voltage of half-maximum activation, \( V_{1/2} \), 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 µM cAMP of -97.9±1.9 mV. Thus, the steady-state activation was shifted by 19.9±1.2 mV to more depolarized values due to cAMP binding (Figure 1D). The slopes of 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 \( \tau_{a,conc} \), decreased with increasing cAMP concentrations (Figure 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 (Figure 1F).

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

We tested three cAMP-derivatives, used as PKA activators, regarding their ability to activate HCN2 channels: N6-Phe-cAMP, N6-Bn-cAMP, and N6-Bnz-cAMP (Figure 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 N6-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 Figure 2B. All three derivatives caused an increase of the current amplitude and an acceleration of the activation kinetics, as it is known for cAMP (see Figure 1).

cAMP modifications at position N6 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 (Figure 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 EC50 and Hill coefficient, H, are summarized in Figure 2D and E, respectively. The nature of the N6 modification had a major influence on the apparent affinity (EC50,Phe = 3.02±0.83 nM, EC50,Bn = 5.12±1.03 nM, EC50,Bnz = 374±91.3 nM) (Figure 2D). Adding a phenyl or benzyl group to position N6 shifted the EC50 value to lower concentrations compared to native cAMP, causing an apparent affinity one order of magnitude higher than for cAMP. However, adding a benzoyl group shifted the EC50 value to higher concentrations, indicating an apparent affinity one order of magnitude lower than for cAMP (Figure 2D). The Hill coefficients for the derivatives were not significantly different from that for cAMP: H_Phe = 1.4±0.3, H_Bn = 1.2±0.3, H_Bnz = 0.9±0.2 (Student’s t-test, p=0.05) (Figure 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: (1) the maximal agonist-induced voltage shift (∆V1/2,max) and (2) the maximal fractional increase in tail current amplitude (I_max,agonist / I_max,cAMP) observed with saturating agonist concentrations (Figure 3) (30). To yield the maximal agonist-induced voltage shift (∆V1/2,max), V1/2 values before and after application of saturating agonist concentrations were estimated by fitting the Boltzmann equation to steady-state activation relationships (Figure 3A). There was no difference between ∆V1/2,max of cAMP and either one of the tested derivatives (16.1±2.3 mV for cAMP, 18.3±1.4 mV for N6-Phe-cAMP, 18.6±2.4 mV for N6-Bn-cAMP, and 22.3±1.1 mV for N6-Bnz-cAMP) (Figure 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 µM 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 (Figure 3C). The values were 1.02±0.14 for N6-Phe-cAMP, 0.98±0.05 for N6-Bn-cAMP, and 1.00±0.02 for N6-Bnz-cAMP (Figure 3B).

Thus, both measures led us conclude that the modifications performed in the tested cAMP derivatives did not affect the efficacy of the agonists.

None of the cAMP modifications tested changed activation kinetics.

Figure 4 summarizes the results regarding activation kinetics upon hyperpolarizing
voltage jumps. First, we studied the concentration dependence of this 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 $\tau_{a,\text{conc}}$ 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 $[\text{agonist}]/EC_{50}$ (Figure 4A). Such normalization was required because large differences in the $EC_{50}$ values for the derivatives and cAMP did not allow for a comparison at absolute concentrations. As a result, the concentration dependence of $\tau_{a,\text{conc}}$ 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 Figure 4B. Analogous to the concentration dependence, the voltage dependence of $\tau_{a,\text{voltage}}$ could be superimposed with that of 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 R635 for determining the binding behaviour of N$^6$-modified derivatives.** To determine the structural basis for 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 (Figure 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 (Figure 5A), indicating that 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; Figure 5B, 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 to 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 R635 (Figure 5A, B, 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 due to the more restricted conformational freedom of the N$^6$-ring bonds compared to the less restricted N$^6$-benzyl carbon bond in N$^6$-Bn-cAMP, which can explain the higher apparent affinity of N$^6$-Phe-cAMP compared to 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: 8.66 Å) but was instead inverted (Figure 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. Due to 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 R635 (Figure 5E), resulting in a strongly disfavorable interaction.
Substituting R635 at the entrance of the binding pocket prevented the higher apparent affinity of N⁶-Phe-cAMP.

The arginine at position 635 was substituted to either alanine for neutralization (R635A) or glutamate for charge reversal (R635E). If R635 is indeed involved in the higher apparent affinity of N⁶-Phe-cAMP and N⁶-Bn-cAMP, the mutations should cause an apparent affinity that resembles that of cAMP to wild type channels. To test this, the concentration-activation relationship was tested for N⁶-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 if those constructs are still reactive to 10 µM cAMP. As for wild type channels we found a substantial current increase and an acceleration of the activation speed (Figure 6A). When studying the concentration-response relationships for N⁶-Phe-cAMP, we found, as expected, that for both constructs the EC₅₀ values were increased (Figure 6B, C): 27.7±5.5 nM for R635A and 33.9±5.3 nM for R635E. They are not different anymore to EC₅₀ values found for cAMP (21.3±3.3 nM) in wildtype channels.

DISCUSSION

PKA-agonists activate HCN2 channels

Herein, we performed a comparative study of three cAMP derivatives, N⁶-Phe-cAMP, N⁶-Bn-cAMP, and N⁶-Bnz-cAMP, earlier described as activators of the protein kinase A (PKA), to investigate their effects on HCN2 channels. Within the last decades, these derivatives have been shown to be useful tools when discrimination between activation of PKA and the exchange protein directly activated by cAMP (Epac) was required (18-22). Interestingly, all three tested derivatives turned out to be also 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 the concentration of half-maximum activation, EC₅₀, as a measure of the apparent affinity, and the Hill coefficient of activation, H. The apparent affinity was considerably affected by the type of N⁶-modification: Substituting one of the N⁶-bound hydrogens by a phenyl- or benzyl group dramatically increased the apparent affinity (seven times and four times higher than cAMP, respectively), whereas a benzoylring decreased it (18 times lower than cAMP). However, because EC₅₀ is a function of both binding affinity and efficacy, this parameter alone does not allow for any interpretation about the mechanisms behind the agonist-dependent differences. The Hill coefficients (Hill, 1910) for all agonists were similar to the Hill coefficient for cAMP. Even though this parameter was 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).

To address the question why the tested agonists show those clear differences in their apparent affinities, we determined the efficacy, the ability of a ligand to elicit a response upon binding to a receptor (32), in comparison to unmodified cAMP. Two parameters were quantified: (1) the maximum extent of shifting the steady state activation to more depolarized voltages, ΔV₁/₂, and (2) the ability of increasing the current in channels maximally activated by voltage. For both parameters, none of the three agonists differed from cAMP, indicating a similar efficacy for the native ligand and the three
derivatives. Following a definition formulated by Colquhoun (1998) (31), according to which efficacy is determined by the sum of transduction events that follow the initial binding reaction, in HCN2 channels those transduction events are similar no matter if they bind N^6-Phe-cAMP, N^6-Bn-cAMP, N^6-Bnz-cAMP or the native ligand cAMP. Provided that cAMP is a full agonist in HCN2 channels (33) those data further suggest considering all three N^6-modified derivatives also as full agonists.

Additionally 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 reflects the kinetics of the underlying channels and is 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: (1) at varying voltages in the presence of a saturating agonist concentration, and (2) at varying concentrations at a saturating voltage. For both conditions the activation time constants for each of the three derivatives was not different from the activation time constants for cAMP. This supports the interpretation of the 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 herein are differently 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 R635. The higher apparent affinity of N^6-Phe-cAMP compared to 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 phenyl ring and R635 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 R635 is the main cause for 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 a apparent affinity of N^6-Phe-cAMP similar to the apparent affinity of cAMP for mHCN2 wildtype channels. From this result, we suggest that now N^6-Phe-cAMP interacts with R635A in a similar manner as cAMP with the mHCN2 wildtype. Reversing the charge by replacing the arginine with a glutamate led to an apparent affinity of N^6-Phe-cAMP similar to the apparent affinity of cAMP for mHCN2 wildtype channels. From this result, we suggest that now N^6-Phe-cAMP interacts with R635A in a similar manner as cAMP with the mHCN2 wildtype. 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 wildtype 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 R635. A role of R635 for binding and selectivity was discussed earlier by Zhou and Siegelbaum (2007)
(30). They described this residue as being involved in discriminating between cAMP and cGMP without contributing to the efficacy.

**Usability of N⁶-modifications in cAMP to discriminate between PKA and HCN channels**

What can we learn from the results presented herein 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 EC₅₀ values for PKAI and PKAII obtained from the literature (EC₅₀/cAMP) versus relative EC₅₀ values for HCN2 obtained herein (Figure 7) (29,34-36).

The very high apparent affinity of N⁶-Phe-cAMP as well as the very low one of N⁶-Bnz-cAMP for HCN2 channels brings the N⁶ position into focus for the search of discriminating agonists. However, in the case of N⁶-Phe-cAMP, also the apparent affinity for PKA I and PKAII was shown to be relatively high, with a factor of ~2.4 and ~2.8 higher for PKAI and PKAII, respectively, compared to cAMP (29). Thus, the discrimination between HCN and PKA is not very strong.

N⁶-Bn-cAMP was also very potent for HCN2 channels, while it was in most cases less potent than cAMP in PKA I (29,34,36). The differences are not very high; however, a benzylring in N⁶ position could be at least one of several modifications in the cAMP molecule to yield discrimination between HCN2 and PKAI. A discrimination to PKAII is questionable, because the apparent affinity was shown to be either unchanged (29) or two times better than for cAMP (36). The derivative with the best discrimination ability found herein is N⁶-Bnz-cAMP. It is a potent agonist for PKAI with a similar apparent affinity as cAMP (29,35), but a relatively poor agonist for HCN2 with an apparent affinity eighteen times worse than cAMP. This is a very important finding because N⁶-Bnz-cAMP is a well-established tool for studying cAMP mediated signal transduction in a wide range of cellular processes (e.g. (23,37,38)). Discrimination between PKAII and HCN2 is expected to be less strong, though, because N⁶-Bnz-cAMP is two times less potent to PKAII than cAMP (29,35).

The C-helix of CNBDs (green bar in Figure 7) acts as a hydrophobic lid covering the base moiety of the cyclic nucleotide and thereby capping the binding pocket (39). Is the positively charged arginine R635 at the C-terminal end of this C-helix a unique feature for HCN channels and can it be used for further discrimination approaches? The alignment in Figure 7 shows that, with the exception of mHCN4, R635 is highly conserved in mouse and human HCN channel isoforms, which allows for speculating of an important role in ligand binding. The comparison with A- and B sites of PKAI and PKA II shows that there is no such arginine at the C-terminal ends of the PKA C-helices. However, there is an interesting pattern of charged amino acids right before R635, which reads negative-positive-X-negative-positive and can be found in most HCN channels. This pattern is also present in A sites of PKA RI and, slightly modified, in PKA RII, although not as part of the C-helix sequence but in the consecutive loop directly behind. Such a pattern could not be found in any B sites of PKA presented here. Another pattern in HCN CNBDs is a strong positive spot at the N-terminal part of the C-helix followed by negative residues two positions C-terminal from that. Again this pattern can be found in A sites of PKA RI and additionally in RII, but not in B sites. Those differences might be responsible for N⁶-Phe-cAMP, N⁶-Bn-cAMP, and N⁶-Bnz-cAMP preferring A sites over B sites (34,41).
Because the C-helix is the most variable structure of CNBDs (39), residues in that helix are potential key players in determining protein-specific ligand selectivity. After comparing sequences of HCN CNBDs with A sites and B sites, we speculate that there is a greater similarity between binding to HCN CNBDs and A sites than between HCN CNBDs and B sites. Thus, for identifying further cAMP derivatives with a higher degree of discrimination between HCN and PKA than shown herein we propose to focus on the unique characteristics of binding to B sites.

In summary, our analysis shows that a possible cross-activation of CN-modulated ion channels should be taken into account when CN-dependent cellular processes are investigated with N6-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

Xenopus laevis oocytes as heterologous expression system
The surgical removal of oocytes was performed under anaesthesia (0.3% tricaine (MS-222) (Pharmaq Ltd. Fordingbridge, UK) from adult females of the South African claw frog Xenopus laevis. The oocytes were treated with collagenase A (3 mg/ml; Roche, Grenzach-Wyhlen, Germany) for 105 min in Ca2+-free Barth’s solution containing (in mM) 82.5 NaCl, 2 KCl, 1 MgCl2, and 5 Hepes, 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 (in mM) 84 NaCl, 1 KCl, 2.4 NaHCO3, 0.82 MgSO4, 0.41 CaCl2, 0.33 Ca(NO3)2, 7.5 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 lab 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'-TCATACTCGCGCGGCACCCCAAGGT TTC-3' (restriction site MauBI, outer primer) and reverse 5'-CTTCTTGCTATCTCATCTAGCCGGT CAATAGC-3' (R635E) or 5'-CTTCTTGCTATGCGATCTAGCCGGT CAATAGC -3' (R635A). PCR 2: forward 5'-
GCTATTGACCGGCTAGATGAGATAG GCAAGAAG-3' (R635E) or 5'-
GCTATTGACCGGCTAGATGCGATGCAAGAAG 3' (R635A) and reverse 5'-
AGCAGGGTTGGTCTAGAGTCACAAG TTGGAAGAG-3' (restriction site XbaI, outer primer). In a third PCR the PCR products of PCR 1 & 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 run down phenomena. Patch pipettes were pulled from quartz tubings whose outer and inner diameter were 1.0 and 0.7 mm (VITROCOM, New Jersey, USA), respectively, using a laser puller (P-2000, Sutter Instrument, Novato, USA). The pipette resistance was 1.2-2.1 MΩ. The bath solution contained (in mM) 100 KCl, 10 EGTA, and 10 Hepes, pH 7.2, and the pipette solution contained (in mM) 120 KCl, 10 Hepes, and 1.0 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, Holliston, US) was used for current recording. Pulsing and data recording were controlled by the Patchmaster software (Harvard Apparatus, Holliston, US). The sampling rate was 5 kHz. The holding potential was generally -30 mV.

**Quantification and Statistical Analysis**

Concentration-activation relationships were analysed by approximating the Hill equation to each individual recording using the OriginPro 9.0G software (Northampton, USA).

\[ \frac{I}{I_{\text{max}}} = \frac{1}{1 + \left( \frac{EC_{50}}{[\text{agonist}]} \right)^H} \]  
(equation 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 analysed by fitting the Boltzmann equation to each individual recording using the OriginPro 9.0G software (Northampton, USA).

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

\[ I(t) = A \exp\left[ -\frac{t}{\tau} \right] \]  
(equation 3)

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

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

**Molecular modelling 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 mHCN2J (25) was prepared using the Protein Preparation Wizard in Maestro; the program was evoked to cap the termini with ACE/NMA (acetyl and N-methyl amide) moieties and to convert selenomethionine residues back to methionine residues. The potential grid for
docking was centered on the co-crystallized cAMP and the ligand length was set to $\leq 16$ Å. The dimensions of the inner box that restricts the region in which the diameter midpoint of each ligand can be located were set to $10 \times 10 \times 10$ 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” (XP) (26) mode with default options.

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CONFLICT OF INTEREST
F.S. is Head of Research & Development of BIOLOG Life Science Institute that sells cAMP analogues that were used in this study.

AUTHOR CONTRIBUTIONS
T.L and F.Sp. carried out the electrophysiological measurements and analysed the data. M.B. and H.G. carried out the molecular modeling and docking experiments, analysed the data, prepared the figures, and wrote the manuscript. F.S. synthesized the compounds. T.S. engineered the mutants. J.K. designed the electrophysiological experiments, analysed the data, prepared the figures, and wrote the manuscript.

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FIGURE LEGENDS

Figure 1. cAMP effects on steady-state and non-steady 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 (exemplary for a saturating concentration of 10 µM 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 relative current amplitudes (I/I_{max}) were obtained from 6 to 13 recordings and plotted against the cAMP concentration. The Hill equation (equation 1) was approximated to the data, yielding the concentration of half-maximum activation, EC_{50} (21.3±3.3 nM), and the Hill coefficient \(H\) (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 µM (n=13) were plotted against the command voltage. The Boltzmann equation was approximated to the data, yielding \(V_{1/2}=-117.8±1.5\) mV and a slope of \(z\delta=4.2±0.3\) for zero and \(V_{1/2}=-97.9±1.9\) mV and a slope of \(z\delta=4.2±0.3\) for 10 µM cAMP. E) Activation kinetics at different cAMP concentrations. Activation 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 3 to 7 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 µM cAMP. The protocol used is shown in B).

Figure 2. Structure and apparent affinities of the three tested derivatives.
A) Molecular formulas for three N⁶-modified derivatives known to activate PKA. B) Representative current responses before and after application of the respective ligand. Black traces represent recordings in the absence of the ligand, colored traces represent recordings during application of a saturating concentration of the respective ligand. Each ligand turned out to be an activator of heterologously expressed HCN2 channels. C) Concentration-response relationships for the three derivatives (colored symbols and fits) in comparison to mHCN2 wildtype data (black symbols and fits). The Hill equation (equation 1) was approximated to the data to obtain the half-maximum concentration, EC_{50}, and the Hill coefficient, \(H\). Error bars indicate S.E.M. D) Box plot of EC_{50} values obtained from C. Filled circles indicate individual recordings. Error bars indicate S.D. E) Box plot of Hill coefficients obtained from C. Filled circles indicate individual recordings. Error bars indicate S.D.

Figure 3. Efficacy of the three tested derivatives.
A) Steady-state activation relationships at zero and saturating agonist concentrations. The graphs show the relationships for the tested derivatives at saturating concentrations and in the absence of any ligand. Except for N⁶-Bnz-cAMP, for which 100 µM was used as saturating concentration, the concentration was 10 µM for saturation. The relative currents (I/I_{max}) are mean values obtained from 3 to 6 recordings. The Boltzmann equation was approximated to the relative current values to yield \(V_{1/2}\). The results of the fits were the following: -97.5±2.0 mV for N⁶-Phe-cAMP, -104.9±2.7 mV for N⁶-Bn-cAMP, and -98.3±1.4 mV for N⁶-Bnz-
cAMP. The slopes were 3.4±0.2, 4.1±0.2, and 4.4±0.3 respectively. B, C) Comparison of the efficiency of the three derivatives in comparison to cAMP in two measures. B) Box plots of relative maximum current amplitude $I_{\text{max}} / I_{\text{max, cAMP}}$. Filled circles indicate individual recordings. Error bars indicate S.D. C) Box plots of maximum agonist-induced shift of half-maximum voltage $\Delta V_{1/2}$. 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. $\tau_{a,\text{conc}}$ values, obtained from approximating a monoexponential equation (Equation 3) to the current time courses, are plotted against the cAMP concentration. Mean values were obtained from 3 to 9 recordings. Black symbols in each plot illustrate the case for cAMP. The protocol used is shown in 1A). B) Activation kinetics in dependence on command voltage at a saturating agonist concentration. $\tau_{a,\text{voltage}}$ values were plotted against the command voltage. Open symbols represent recordings in the absence of ligands, filled symbols in the presence of saturating agonist concentrations. Mean values were obtained from 3 to 19 recordings. The protocol used is shown in 1B).

**Figure 5. Predicted binding modes of the tested cAMP-derivatives.**
Predicted binding modes of A) cAMP (grey); crystallographic pose (PDB ID: 1Q5O (25) shown in gold, B) N$^6$-Phe-cAMP (blue), C) N$^6$-Bn-cAMP (orange), and D) N$^6$-Bnz-cAMP (magenta). In panel 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-$\pi$ 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 R635 resulting from this extension is highlighted (red box), explaining the inverted binding mode of N$^6$-Bnz-cAMP shown in panel D).

**Figure 6. Response to N$^6$-Phe-cAMP after charge neutralization or reversal at position R635 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 t-test).

**Figure 7. Comparison of relative apparent affinity for PKA and HCN2.**
A) Apparent affinities for cAMP derivatives were related to apparent affinities for unmodified cAMP. Amongst the tested derivatives the best discrimination can be realized with N$^6$-Bnz-cAMP. Data for PKA were obtained from: (29,34-36). Filled symbols represent PKAI, empty symbols PKAII. B) Sequence alignment comparing the distal parts of the C-helix sequences of mouse and human HCN isoforms with those from A and B sites in regulatory subunits of human PKAI and II. Alignments and structure predictions ($\alpha$-helix C marked in green) are shown as proposed by Berman and co-workers (2005) (39), who used a structure and transformation method (SAT). Prediction of the HCN C-helix follows a suggestion of Lee and MacKinnon (2017) (40). The asterisks mark R635 mutated herein. Positively charged residues are shown in red, negatively charged in blue.
Figure 1
Figure 2
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Figure 7
N\textsuperscript{6}-modified cAMP derivatives that activate protein kinase A also act as full agonists of murine HCN2 channels
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