Type-specific Regulation of Adenylyl Cyclase

SELECTIVE PHARMACOLOGICAL STIMULATION AND INHIBITION OF ADENYLYL CYCLASE ISOFORMS*

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Crystallographic studies have elucidated the binding mechanism of forskolin and P-site inhibitors to adenylyl cyclase. Accordingly, computer-assisted drug design has enabled us to identify isoform-selective regulators of adenylyl cyclase. After examining more than 200 newly synthesized derivatives of forskolin, we found that the modification at the positions of C6 and C7, in general, enhances isoform selectivity. The 6-(3-dimethylamino-propionyl) modification led to an enhanced selectivity for type V, whereas 6-[N-(2-isothiocyanatoethyl) amino-carbonyl] and 6-(4-acrylbutyryl) modification led to an enhanced selectivity for type II. In contrast, 2'-deoxyadenosine 3'-monophosphate, a classical and 3'-phosphate-substituted P-site inhibitor, demonstrated a 27-fold selectivity for inhibiting type V relative to type II, whereas 9-(tetrahydro-2-furyl) adenine, a ribose-substituted P-site ligand, showed a markedly increased, 130-fold selectivity for inhibiting type V. Consequently, on the basis of the pharmacophore analysis of 9-(tetrahydro-2-furyl) adenine and adenylyl cyclase, a novel non-nucleoside inhibitor, 2-amino-7-(2-furanyl)-7,8-dihydro-5(6H)-quinazolinone (NKY80), was identified after virtual screening of more than 850,000 compounds. NKY80 demonstrated a 210-fold selectivity for inhibiting type V relative to type II. More importantly, the combination of a type III-selective forskolin derivative and 9-(tetrahydro-2-furyl) adenine or NKY80 demonstrated a further enhanced selectivity for type III stimulation over other isoforms. Our data suggest the feasibility of adenylyl cyclase isoform-targeted regulation of cyclic AMP signaling by pharmacological reagents, either alone or in combination.

The G protein1-sensitive, membrane-bound form of adenylyl cyclase consists of a large family; nine isoforms have been isolated and extensively studied (1–3). These isoforms are characterized by distinct biochemical properties and tissue distribution. For example, calcium-inhibitable type V is expressed in the heart as a major isoform (4); protein kinase C-sensitive type II is expressed in lungs (5); calmodulin-sensitive type I and type VIII are expressed exclusively in neuronal tissues (6); type III is expressed abundantly in olfactory tissues (7, 8) but also in other tissues including lungs (9), atria (10), and adipose tissue (11); type IV and VII are widely expressed (12, 13). Therefore, it is now accepted that the content and mixture of adenylyl cyclase isoforms provide a biochemical signature of tissue cyclic AMP generation.

Forskolin, like digitalis, is a natural plant extract that has been used in traditional medicine (14). Forskolin directly activates adenylyl cyclase to increase the concentration of intracellular cyclic AMP. This mechanism for activation is now explained as follows. Forskolin binds to the catalytic core at the opposite end of the same ventral cleft that contains the active site and activates the enzyme by gluing together the two cytoplasmic domains in the core (C1 and C2) using a combination of hydrophobic and hydrogen bond interactions (15). Although the efficacy of forskolin was confirmed in human studies (16, 17), its poor tissue selectivity has hampered its clinical use. Recently, however, a water-soluble forskolin derivative 6-[3-(dimethylamino)propionyl]forskolin (NKH477) was introduced to treat human heart failure (18, 19). NKH477 is a forskolin derivative in which a 3-(dimethylamino)propionyl group was attached to forskolin at the C6 position. Furthermore, NKH477 was found to have enhanced type V selectivity (20). As predicted by a recent crystallographic study, there is a relatively large open space between the C6/C7 positions of forskolin and its binding site within adenylyl cyclase (15, 21), implying that a forskolin derivative modified in these positions might have altered isoform selectivity without disrupting their activity; this is consistent with the findings of NKH477 (20).

In contrast, P-site inhibitors are adenosine analogs that inhibit adenylyl cyclase (22). P-site inhibitors bind to the same binding site as the substrate ATP within the adenylyl cyclase molecule (23); as yet the mode of inhibition is either un- or non-competitive with respect to ATP as shown by kinetic analysis (24). P-site inhibitors occupy the site where cyclic AMP is accommodated, forming a dead-end complex with pyrophosphate (25, 26). Most importantly, a recent study demonstrated...
selective inhibition of adenylyl cyclase isoforms by certain P-site inhibitors (27). These findings suggested that P-site ligands can serve as isoform- and, therefore, tissue-selective regulators of cyclic AMP signaling. A major concern, however, is that P-site ligands require the presence of an intact adenine ring moiety to retain inhibition. Such molecules might therefore be expected to lack specificity and affect other pathways within the cells, including DNA synthesis. Indeed, a recent study demonstrated that acyclic derivatives of adenine possess both antiviral and adenylyl cyclase inhibitory effects (28).

The above findings have prompted us to search for forskolin derivatives with enhanced isoform selectivity and non-nucleoside inhibitors that may mimic P-site ligands. Based upon the findings from a prior crystallographic study (15) and computer-assisted drug design, we have identified forskolin derivatives that have enhanced selectivity for type II, type III, and type V, respectively. Furthermore, we have found a novel non-nucleoside inhibitor of the type V isoform, which was obtained after virtual screening of more than 850,000 compounds on the basis of the pharmacophore analysis of adenylyl cyclase and P-site ligands. We have also found enhanced selectivity in regulating tissue adenylyl cyclase catalytic activity with the use of these compounds.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Forskolin, 2'-d-3'-AMP, 3'-AMP, Ap(Ch)2ppp, and GTP•S were purchased from Sigma. More than 200 forskolin derivatives, 9-(tetrahydro-2-furyl)adenine (THFA or SQ 22,536), 9-(cyclopentyl)adenine (CPA), and N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) were purchased from Research Biochemicals International (Natick, MA). Phenylcyclopentyl)-azacyclopentadec-1-en-2-amine (MDL 12330A) was purchased from Biomol Research Lab., Inc. (Plymouth Meeting, PA).

**Overexpression of the Adenylyl Cyclase Isoforms and Gα in Insect Cells**

Overexpression of each adenylyl cyclase isoform and Gα were performed as previously described (20). High Five cells were washed twice with ice-cold phosphate-buffered saline and homogenized in a buffer containing 50 mM Tris/HCl (pH 8.0), 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 200 mM sucrose, and a protease inhibitor mixture containing 20 μg/ml 1-chloro-3-tosylamido-2-heptanone, 10 μM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, 50 μl/ml egg white trypsin inhibitor, and 2 μg/ml aprotinin (Buffer A). Cells were disrupted with a sonicator and centrifuged at 500 × g for 10 min at 4 °C. The supernatants were further centrifuged at 100,000 × g for 40 min at 4 °C. The resultant pellets were resuspended in the same buffer without EDTA (Buffer B). For Gα preparation, we used the supernatant fraction after ultracentrifugation. The crude membrane and the Gα-rich supernatant were stored at −80 °C until use. Protein concentration was measured with the Bio-Rad protein assay system.

The overexpression of recombinant adenylyl cyclase isoform in insect cells increased catalytic activity by ~56-fold for type II, 48-fold for type III, and 360-fold for type V over that of control cell membranes as determined in the presence of Gαi-GTP•S-forskolin (50 μM). Thus under these conditions, each isoform was examined as the dominant positive adenylyl cyclase isoform in these cells.

**Tissue Preparation**

Male Wistar rats were purchased from Charles River Japan, Inc. (Yokohama, Japan). Tissues were minced and homogenized with a Polytron for 3 × 10 s in Buffer A followed by centrifugation at 500 × g for 10 min at 4 °C. The supernatants were retained and further centrifuged at 100,000 × g for 40 min at 4 °C. The crude membrane preparations were made by resuspending the pellet in Buffer B and stored at −80 °C until use. Animals used in this study were maintained in accordance with the guidelines of the animal experiment committee of the Yokohama City University School of Medicine.

**Adenylyl Cyclase Assay**

Adenylyl cyclase catalytic activity was measured as previously described with some modification (29). Briefly, the reaction mixtures contained 20 mM HEPE (pH 8.0), 0.5 mM EDTA, 0.1 mM ATP containing (α-32P)ATP (1 × 106 cpm), 0.1 mM cyclic AMP, 1 mM creatine phosphate, 8 units/ml creatine phosphokinase, 5 mM MgCl2 or 15 mM MnCl2, and 4 μg (for insect cells) or 8 μg (for tissues) of membrane protein in a final volume of 100 μl. Gαi-enriched supernatant obtained from insect cell overexpressing Gα was used in an amount that stimulated adenylyl cyclase maximally in the presence of 1 μM GTP•S. Further details are provided in the figure legends.

Assays were performed at 30 °C for 20 min and terminated by the addition of 10 μl of ice-cold 2.2 × HCl. The product 32P-cyclic AMP was separated with single acidic alumina columns (30). In brief, the samples were applied to the acidic alumina column (ICN Pharmaceuticals, Inc., Costa Mesa, CA) and washed with 0.005 n HCl to remove any unbound contaminants. The bound cyclic AMP was eluted with 0.1 n ammonium acetate (pH 7.0). The radioactivity of the eluted samples was measured by scintillation counting. Determinations of sample recovery using [3H]-cyclic AMP were omitted because high cyclic AMP recovery (86–93%) was typically achieved with this system in a validation study, and the data were similar to that obtained using the conventional two-column method (31). Results were obtained from quadruplicate determinations unless specified and are shown as the means ± S.D.

**Virtual Docking and Screening Study**

**Modeling—**Amino acid sequences of rat adenylyl cyclase type II and type III and canine adenylyl cyclase type V were obtained from sequence data bases (PIR and SWISSPROT). The three-dimensional structures of adenylyl cyclases were modeled using the homology modeling method. The crystal structure of the catalytic core of adenylyl cyclase, which consists of the two homologous cytoplasmic domains (Cα and Cβ), was used as a template. In this structure the Cα and the Cβ domains were those from canine type V and rat type II, respectively. The coordinates of the crystal structure were retrieved from the Protein Data Bank (entry: 1AZS). The sequences of the three isoforms of adenylyl cyclase were aligned for 1AZS with Chem-X (Chemical Design Ltd., Oxon, England) using default parameters. Because there is no atomic coordinates for the structures between PRO:B954 and GLU: B963 of the Cα domain of 1AZS, the region was removed from the alignment. As a result, there is neither gap nor insertion in the alignment between the target sequences and the template sequence. Therefore, the amino acids of the template were simply mutated into those of the target at different amino acid sites using Chem-X without altering the backbone conformation. Hydrogen atoms were added to the model in Insight II 98.0 (Molecular Simulations Inc., San Diego, CA).

**Refinement—**As the first step of refinement, all close contacts caused by the mutation of side chains were fixed by searching the most suitable conformer of the side chains from the established rotamer libraries of Biopolymer module within Insight II. The model was relaxed by energy minimization using Discover with the force-field van der Waals and electrostatic solvent force field according to the following protocol: (i) minimization of all hydrogen atoms with all heavy atoms fixed, (ii) minimization of the side chains of mutated residues with main chain fixed, (iii) minimization of all the side chains with main chain fixed, (iv) minimization of the whole system using a harmonic force constant of 10 kcal/mol Å on all the target and different amino acid sites using Chem-X without altering the backbone conformation. Hydrogen atoms were added as a convergence criteria with a dielectric constant of 4.

**Docking—**Forskolin derivatives were manually docked into the binding site guided by an overlay of the fused rings onto forskolin in the pocket of adenylyl cyclase from the crystal structure. After the minimization of the complex of a forskolin derivative with adenylyl cyclase, water molecules were added within a sphere of 25 Å from the center of the forskolin derivative. The solvated system was minimized, and then the MD simulation at 298 K was performed with Discover to search the low energy docking mode.

**Virtual Screening—**Based upon a previous study in which the interaction of 2'-d-3'-AMP to adenylyl cyclase was examined (21), we used a pharmacophore (−C=H−N=C(NH2)−) that we presumed necessary for the inhibition of adenylyl cyclase in our virtual screening. More than 850,000 chemical compounds available from an existing data base were screened using ISIS (MDL Information Systems Inc., San Leandro, CA), and potential candidates were subjected to adenylyl cyclase assays. The pharmacophore structure (−C=H−N=C(NH2)−) of the identified compounds was then superimposed on that of 2'-d-3'-AMP in the crystal.
structure of the complex consisting of adenylyl cyclase and 2'-d-3'-AMP followed by minimization of the complex consisting of the type V enzyme and the compound using Discover.

RESULTS

Effects of Modifying Different Positions of Forskolin—As to the isoform selectivity of adenylyl cyclase, there has been little data available about the structure/function relationship of forskolin. Therefore, to develop an approach for modifying forskolin that might increase isoform selectivity, we synthesized more than 200 derivatives of forskolin that were modified at the positions of C1, C6, C7, C9, C11, and C13 and examined their effects on the catalytic activity of the adenylyl cyclase type II, type III, and type V, which belong to different subgroups within the adenylyl cyclase family. The relative stimulatory activity of each derivative versus forskolin (% forskolin activity) is shown in the following results. It is important to note that most of the newly synthesized forskolin derivatives either had no isoform selectivity or lost their ability to stimulate adenylyl cyclase.

In general, the modification at the C1, C9, C11, and C13 positions of forskolin resulted in loss of adenylyl cyclase stimulatory activity, whereas a small enhancement in isoform selectivity was noted in a few cases. An example was 1,9-dideoxyforskolin, which is known to be an inactive forskolin derivative (32, 33). In contrast, 11-deoxy-11-hydroxyforskolin was a weak stimulator and had a small enhancement in isoform selectivity; the relative potency of stimulating each isoform versus forskolin (% forskolin activity) was 44% for type II, 19% for type III, and 55% for type V. Forskolin derivatives that were substituted with an alkyl group at the C13 position such as 13-devinyl-13-propionylforskolin, 13-devinyl-13-hydroxymethylforskolin, and 14,15-dihydro-15-chloroforskolin were mostly inactive on any isoform. An exception was that with an unsubstituted ethyl group at the position C15 (14,15-dihydroforskolin), which has been used in radioligand binding assays (34). It exhibited decreased stimulatory activity with a small enhancement in isoform selectivity; the relative potency of stimulation versus forskolin was 39% for type II, 25% for type III, and 41% for type V.

Forskolin Derivatives with Enhanced Type II Selectivity—As shown in a previous crystallographic study (21), a relatively large open packing space exists between adenylyl cyclase and the C6 position of forskolin, whereas a tight hydrogen bond exists between adenylyl cyclase and other positions of forskolin such as the hydrogen group of C1 and the carbonyl group of C11. There is also a tight hydrogen bond between the carbonyl group of C7 and adenylyl cyclase; however, there exists an open packing space between the methyl group of C7 and adenylyl cyclase. Thus, we thought that modification of these residues (C6 and C7, see Table I), unlike the earlier modifications, may promote isoform selectivity without loss of potency. Indeed, that was the case. Results from representative derivatives (FD1-FD6) are summarized in Table I and Fig. 1.

6-[N-(2-Isothiocyanatoethyl)aminocarbonyl]forskolin (FD1) was originally reported to irreversibly inhibit forskolin binding to the type I isoform of adenylyl cyclase; its effect on the other isoforms remained unexamined (35). We found that this derivative exhibited enhanced stimulation of type II, whereas it very weakly stimulated type III and type V; the relative potency of stimulation of this derivative versus forskolin was 219% for type II, 46% for type III, and 21% for type V (Fig. 1).

We thus investigated the mechanism that led to increased selectivity for type II. The isothiocyanate group at the C6 position of this derivative can interact with functional groups with high nucleophilicity such as the ε-amino group of lysine or the thiol group of cysteine. To examine whether this isothiocyanate group contributes to increased selectivity for type II, we synthesized forskolin derivatives in which the isothiocyanate group at C6 was inactivated; an example was 6-(2-thio-ureidoethylaminocarbonyl) forskolin. This derivative was still active but lost type II selectivity (57% on type II, 60% on type III, and 92% on type V). We also synthesized a forskolin derivative that had an α,β-unsaturated carbonyl group at the same position (C6), which is functionally similar to the isothiocyanate group (FD2, 6-(4-acrylbutyryl)forskolin) (see Table I). This derivative retained similar type II selectivity (Fig. 1). Furthermore, a docking study of FD1 with different adenylyl cyclase isoforms predicted that Lys<sup>896</sup> of type II, unique to type II, may interact with the isothiocyanate group at the C6 position of this derivative.

These findings suggest that to enhance type II selectivity forskolin needs to be replaced with a functional group at C6 that can productively interact with Lys<sup>896</sup> of type II. A point mutation study of type II adenylyl cyclase at this residue (Lys<sup>896</sup>), however, will be necessary to address this issue directly.

Forskolin Derivatives with Enhanced Type III-Selectivity—We also examined forskolin derivatives that were modified...
at the C7 position with various functional groups. We found that derivatives to which a polar group was attached at the C7 position, i.e., 7-deacetyl-7-hydroxymethyl forskolin (FD3) or 5,6-dehydroxy-7-deacetyl-7-nicotinoylforskolin (FD4), enhanced their selectivity for type III (Table I and Fig. 1). The stimulatory activity of other isoforms (types II and V) remained similar; the relative potency of stimulation of FD4 versus forskolin was 116% for type II, 307% for type III, and 77% for type V. Similarly, dehydroxyl modification at the positions of C5 and C6 also enhanced selectivity for type III (5,6-dehydroforskolin, 86% for type II, 166% for type III, and 78% for type V). We thus speculate that the polar substitution at the C7 position as well as the attachment of carbon-carbon double bonds to the ring core of forskolin (C5 and C6) contributes to type III selectivity.

**Forskolin Derivatives with Enhanced Type V Selectivity**—We previously reported that 6-[3-(dimethylamino)propionyl]forskolin (NKH477, FD5) had enhanced stimulation of type V, whereas the potency of stimulating other isoforms (types II and III) remained similar (Table I and Fig. 1) (20). It should be noted that NKH477 (FD5) is now used to stimulate cardiac adenylyl cyclase in patients with congestive heart failure (19, 36). Several other forskolin derivatives in which a positively charged group such as 3-dimethylamino)propionyl group was attached to the position of C6 or C7 showed a similar enhancement in type V selectivity. Thus, modification of the C6 or the C7 positions with a positively charged residue resulted in enhanced type V selectivity without losing potency for other adenylyl cyclase isoforms.

As previously stated, 14,15-dihydroforskolin has a weak stimulatory effect on adenylyl cyclase but showed a small enhancement in type V selectivity. We thus combined the two modifications; a 3-(dimethylamino)propionyl group was placed at the C6 position of 14,15-dihydroforskolin. The resulting forskolin derivative (FD6, 6-[3-(dimethylamino)propionyl]-14,15-dihydroforskolin) had a further enhancement in selectivity for type V; the relative potency of stimulation of this derivative versus forskolin was 51% for type II, 22% for type III, and 139% for type V (Table I and Fig. 1). Thus, combining the two modifications, i.e., a minor modification at the C13 position and the 3-dimethylaminopropionyl modification at the C6 position, had additive effects in enhancing isoform selectivity. In summary, our findings strongly suggest that the modification of a specific residue(s) of forskolin increases selectivity for different adenylyl cyclase isoforms, and the combination of multiple modifications further enhances isoform selectivity.

**Concentration-response Analysis**—The relative potency of each forskolin derivative (FD1, -4, and -6) in comparison to that of forskolin is shown in Fig. 2. forskolin stimulated each adenylyl cyclase isoform in a concentration-dependent manner. The selectivity of FD1 for type II, FD4 for type III, and FD6 for type V were clearly demonstrated in terms of the degree of maximal stimulation. However, there was no apparent shift of the curve to the left in any of these forskolin derivatives.

**Inhibition of Adenylyl Cyclase Isoforms by P-site Ligands**—Known inhibitors of adenylyl cyclase include adenosine analogs or P-site inhibitors, which must have an intact adenine ring (22), and MDL 12330A, a non-nucleoside inhibitor (37). We first examined the inhibitory effect of these compounds on the isoforms of adenylyl cyclase in the presence of Gs or GTPγS-forskolin (50 μM). The catalytic activity of each isoform in the presence of each inhibitor (100 μM) was compared with that in the absence of the inhibitors (Fig. 3).

Classic P-site inhibitors with phosphate at the 3' position such as 2'-d3'-AMP and 3'-AMP potently inhibited adenylyl cyclase catalytic activity (Fig. 3). 2'-d3'-AMP potently inhibited type V and type III, while to a lesser degree, type II; the selectivity ratio was 27 between type V and type II. The IC50 value for type V was 51% for type II, 166% for type III, and 78% for type V. Simi-

![Diagram](image.png)

**Fig. 2. Concentration-response curves of forskolin, FD1, FD4, and FD6 for adenylyl cyclase (AC) type II, type III, and type V.** Adenylyl cyclase catalytic activity was measured in the presence of 5 mM MgCl2, with various concentrations of forskolin, FD1, FD4, and FD6. The relative stimulatory activity of each derivative versus 10−4 M forskolin (% forskolin activity) is shown. All experiments were repeated two or more times with different batches of membranes with similar results.

An inhibitor such as THFA and CPA potently inhibited type V, whereas they inhibited type II and type III only to a modest degree in the presence of GαGTGTPγS-forskolin (50 μM). The IC50 value for each isoform was calculated to be 0.82 μM for type V, 2.8 μM for type III, and 22.4 μM for type II (Fig. 4D). In contrast, ribose-substituted P-site inhibitors such as THFA and CPA potently inhibited type V, whereas they inhibited type II and type III only to a modest degree in the presence of GαGTGTPγS-forskolin (50 μM). The IC50 value for each isoform was calculated to be 0.82 μM for type V, 2.8 μM for type III, and 22.4 μM for type II (Fig. 4D). Similar results were obtained in the presence of forskolin alone (Fig. 4B). It was previously noted that type II adenylyl cyclase was less sensitive to THFA than the other isoforms, giving a selectivity ratio of 1.8 when compared between type VI and type II (27). Our data suggested that the selectivity ratio was even greater (130) between type V and type II. Furthermore, type V selectivity was greater with ribose-substituted P-site ligands than with 3' phosphate P-site ligands.

Importantly, the type V selective inhibition by THFA was not the result of the greater catalytic activity of type V as compared with other isoforms since a similar IC50 value (2.2–6.5 μM) was obtained when type V was stimulated by GαGTGTPγS, GαGTGTPγS-forskolin (50 μM), or forskolin alone (1 μM), which gave different catalytic activities. Our data suggest that P-site inhibitors, most likely P-site inhibitors with modified ribose rings such as THFA and CPA, may serve as selective inhibitors of type V adenylyl cyclase.

A competitive inhibitor of ATP binding, Ap(CH2)pp, did not exhibit selectivity among the adenylyl cyclase isoforms (Figs. 3 and 4). Another inhibitor such as MDL 12330A showed a modest degree of inhibition of type II and type III, whereas a lesser degree of inhibition was found for type V. H-89, a protein kinase A inhibitor, did not exhibit selectivity.

**Screening for Type V Inhibitors without an Adenine Structure**—The above data demonstrated that ribose-substituted P-
site ligands such as THFA and CPA selectively inhibited type V. In the development of therapeutic compounds for use in vivo, however, an intact adenine may lead to undesirable side effects such as the inhibition of DNA synthesis (28). Thus, we tried to find type V inhibitors free of the intact adenine structure.

First, we looked for the pharmacophore within THFA that is essential for the inhibition of type V. We synthesized a P-site inhibitor in which the intact adenine structure was disrupted by modifying the C2 position (2-amino-CPA). As shown in Fig. 3, 2-amino-CPA lost both inhibitory effect and type V selectivity, suggesting the importance of an intact adenine structure at the C2 position. The intact adenine structure at the C6 position may also be essential for inhibiting adenylyl cyclase catalytic activity because a crystallographic study has already shown that the N1 and amino group at the C6 position of the adenine ring bind to Asp1018 and Lys938 (type II) via hydrogen bonding (15).

Taken together, these findings suggested that a portion of the adenine structure at the C2 and the C6 positions may play a key role in inhibiting adenylyl cyclase. Accordingly, we screened 850,000 compounds that are commercially available using a pharmacophore screening algorithm and selected 682 compounds that have the pharmacophore in their structure. We then examined 32 representative compounds and identified 2-amino-7-(2-furanyl)-7,8-dihydro-5H-quinazolinone (NKY80) (Fig. 5), which lacks an intact adenine ring yet still inhibited type V in a similar manner to THFA (compare Fig. 4 with Fig. 6). The biochemical characteristics of this compound are summarized in Fig. 6. NKY80, although somewhat less potent, showed a similar type V selectivity to THFA in inhibiting adenylyl cyclase catalytic activity with a selectivity ratio of 210 between type V and type II. The IC50 values were calculated to be 8.3 μM for type V, 132 μM for type III, and 1.7 mM for type II in the presence of Gs·GTP·S-forskolin (50 μM) (Fig. 6A). Similar results were obtained in the presence of forskolin alone (Fig. 6B). A Lineweaver-Burk plot analysis demonstrated that the mode of inhibition of NKY80 was not competitive with respect to ATP (Fig. 6C). Inhibition was also not competitive with respect to forskolin (Fig. 6D).

Tissue-selective Regulation of Cyclic AMP Signal—It is known that each tissue expresses a unique mixture of adenylyl cyclase isoforms (1–3). The adult heart, for example, expresses type V as a major isoform. The lung, on the other hand, does not express type V but, rather, types II and III (5, 9).

We therefore examined the effect of the above forskolin derivatives on tissue adenylyl cyclase catalytic activity from rat heart and lung (Fig. 7). FD6, which has enhanced selectivity for type V, stimulated cardiac adenylyl cyclase more than lung adenylyl cyclase. The specificity of FD6 for cardiac adenylyl cyclase over lung adenylyl cyclase was greater than that of FD5 (NKH477) (data not shown). In contrast, FD4, which has enhanced selectivity for type III, stimulated lung adenylyl cyclase more than cardiac adenylyl cyclase. Thus, our data indicate that each tissue adenylyl cyclase can be stimulated with an enhanced selectivity using a specific forskolin derivative, al-

**Fig. 3. Effect of various inhibitors on adenylyl cyclase type II, type III, and type V.** A, assays were performed in the presence of Gs·GTP·S-forskolin (50 μM) and 5 mM MgCl2 in the presence of various inhibitors. The relative inhibitory activity of these compounds versus control (% control activity) for various adenylyl cyclase isoforms is shown. All experiments were repeated two or more times with different batches of membranes with similar results. B, chemical structure of inhibitors used in A. Cont., control.
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Effect of Divalent Cations—Divalent cations such as Mn$^{2+}$ and Mg$^{2+}$ are essential for catalytic activity, and the degree of activation by divalent cations differs among the adenylyl cyclase isoforms. Forskolin-stimulated catalytic activity of type II was increased by 8.7-fold in the presence of 15 mM Mn$^{2+}$ relative to that in the presence of 5 mM Mg$^{2+}$, that of type III was increased by 3.7-fold, and that of type V was increased by 2.8-fold under the same conditions (Fig. 8). Thus, Mn$^{2+}$ stimulates type II greater than other isoforms. Accordingly, FD1-stimulated catalytic activity of type II was increased by 13.7-fold in the presence of Mn$^{2+}$ relative to Mg$^{2+}$, that of type III was increased by 7.6-fold, and that of type V was increased by 3.3-fold. Thus, type II selectivity of FD1 can be further enhanced in the presence of Mn$^{2+}$ (Fig. 8A). Examination of cardiac and lung adenylyl cyclase catalytic activities revealed similar results (Fig. 8B). Strikingly, FD1-stimulated catalytic activity of lung adenylyl cyclase was increased by 48.5-fold in the presence of Mn$^{2+}$ relative to Mg$^{2+}$, and that of cardiac adenylyl cyclase was increased by only 5.2-fold. Taken together, the above data suggest that the catalytic activity of adenylyl cyclase isoform(s) and, thus, tissue adenylyl cyclase catalytic activity can be selectively increased in vitro by modifying divalent cation conditions in the presence of isoform-selective forskolin derivatives.

Combination of Regulators of Adenylyl Cyclase—We also examined isoform selectivity when an inhibitor and a stimulator
were used together. FD4, as shown earlier, stimulated type III in an isoform-selective manner (Fig. 1). We thus examined the isoform-selective inhibition of THFA in the presence of FD4. As shown in Fig. 9A, the ratio of catalytic activity of type III to type V (type III/type V) increased significantly from 5.5 (when forskolin and THFA were used) to 18.0 (when FD4 and THFA were used). Similar increases were obtained with NKY80 (Fig. 9B). The effects of the above combination were examined on rat lung and cardiac adenylyl cyclase activities (Figs. 9, C and D). With the combination of FD4 and THFA, the ratio of catalytic activity of lung to cardiac adenylyl cyclase (lung/heart) increased significantly from 3.3 (when forskolin and THFA were used) to 4.9 (when FD4 and THFA were used) (Fig. 9C, lower). Results with NKY80 were similar (Fig. 9D). The above findings suggest that the isoform- and, therefore, the tissue-selective regulation of adenylyl cyclase catalytic activity can be further enhanced by the combination of isoform-selective stimulators and inhibitors.

**DISCUSSION**

Our data suggest that it is feasible to target the isoforms of adenylyl cyclase by forskolin derivatives, ribose-substituted P-site ligands, and a novel inhibitor, NKY80, which lacks an intact adenine ring, to regulate cyclic AMP signaling. In particular, our findings suggest that the isoform-selective stimulation of forskolin can be potentiated through specific modifications at the C6 and/or the C7 position of forskolin and that

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**FIG. 7. Effect of forskolin, FD1, FD4, and FD6 on tissue adenylyl cyclases (AC).** Rat tissue membrane preparations were prepared as described under “Materials and Methods.” Tissue adenylyl cyclase catalytic activity was determined in the presence of 5 mM of MgCl2 with forskolin and forskolin derivatives at 100 μM. The relative stimulatory activity of each derivative versus forskolin (% forskolin activity) is shown. Forskolin-stimulated catalytic activities were 93.4 ± 1.3 pmol/min/mg for hearts and 21.6 ± 1.9 pmol/min/mg for lungs. All experiments were repeated two or more times with different batches of membrane and similar results were obtained.

**FIG. 8. Effect of divalent cations on isoform and tissue selectivity.** A, catalytic activity in the presence of Mn2+ over that in the presence of Mg2+ was compared among the isoforms. Catalytic activity in the presence of Mn2+ was 8.7 times that in the presence of Mg2+ for type II, 3.7 times for type III, and 2.8 times for type V when assayed under forskolin-stimulated conditions. When assayed under the FD1-stimulated condition, catalytic activity was 13.7 times for type II, 7.6 times for type III, and 3.3 times for type V catalytic activity. Note that the magnitude of increase is greater for type II and III than type V. B, catalytic activity in the presence of Mn2+ over that in the presence of Mg2+ was compared between lung and cardiac membranes. Catalytic activity in the presence of Mn2+ was 2.6 times that in the presence of Mg2+ for the heart, 19.3 times that for lungs when assayed under forskolin-stimulated conditions. When assayed under the FD1-stimulated condition, catalytic activity was 8.7 times that for heart, 48.5 times that for lungs. Note that the magnitude of increase is greater for lungs than the heart. All experiments were repeated two times with different batches of membranes, and similar results were obtained.

**FIG. 9. Combined effect of stimulator and inhibitor on isoform-and tissue-selective adenylyl cyclase (AC) catalytic activity.** A, combination of FD4 and THFA on adenylyl cyclase isoforms. B, combination of FD4 and NKY80 on adenylyl cyclase isoforms. Adenylyl cyclase catalytic activity was measured in the presence of 5 mM MgCl2 with 50 μM activators (forskolin or FD4) in the presence or absence of 30 μM inhibitor (THFA or NKY80). Upper panel, catalytic activity of type III and type V are shown as the percentage of that in the presence of forskolin alone (% forskolin activity). Lower panel, data are shown as the ratio of type III activity to type V activity (type III/type V activity). All experiments were repeated two or more times with different batches of membranes with similar results. C, combination of FD4 and THFA on tissue adenylyl cyclases. D, combination of FD4 and NKY80 on tissue adenylyl cyclases. Adenylyl cyclase catalytic activity was measured in the presence of 5 mM MgCl2 with 100 μM activators (forskolin or FD4) in the presence or absence of 30 μM inhibitor (THFA or NKY80). Upper panel, catalytic activity of lung or cardiac adenylyl cyclase is shown as the percentage of that in the presence of forskolin alone (% forskolin activity). Lower panel, data are shown as the ratio of lung activity to cardiac activity (lung/heart activity). All experiments were repeated two or more times with different batches of membranes with similar results.
specific effects in various assays (40). Thus, it is tempting to hypothesize that forskolin derivatives do not bind to adenylyl cyclase and have been used as prototypes of forskolin may not necessarily retain the same inter- and the C11 positions may play an important role.

Although forskolin, like P-site ligands, is known to interact with non-adenylyl cyclase molecules in the cell (38, 39), derivatives of forskolin may not necessarily retain the same interaction with such molecules. Indeed, certain forskolin derivatives do not bind to adenylyl cyclase and have been used as tools to differentiate such interactions from adenylyl cyclase-specific effects in various assays (40). Thus, it is tempting to speculate that developing forskolin derivatives that only interact with a specific isoform of adenylyl cyclase may be feasible.

In the present study, however, we neither examined the interaction of our forskolin derivatives to non-adenylyl cyclase molecules nor examined their effects in vivo.

P-site-mediated inhibition has been pharmacologically characterized in detail (15, 21). Crystallographic findings have indicated that the adenine ring of 2′-d-3′-AMP, a classical P-site ligand, binds to Asp1018 and Lys938 in the C2 domain (in type II) via a hydrogen bond in the small hydrophobic pocket and that the phosphate residue of 2′-d-3′-AMP interacts with the P-loop within the C2 domain. Ribose-substituted P-site inhibitors may have lower affinity in comparison to 3′-phosphate P-site inhibitors because they bind only to the C2 domain via the adenine ring, which is in agreement with our findings. Importantly, however, we found ribose-substituted P-site inhibitors have higher type V selectivity. Although we do not know the exact mechanism for this selectivity, differences in the amino acid sequence surrounding Asp1018 and Lys938 in the C2 domain, both of which are conserved in type II and type V, may be responsible for the increased selectivity.

Although both P-site inhibitors and ATP bind to the same site, kinetic analysis of P-site inhibitors has indicated a non- or uncompetitive inhibition with respect to ATP (41). The mechanism underlying this apparent paradox is now explained in that P-site inhibitors and reaction products (cyclic AMP and pyrophosphate) bind to a different adenylyl cyclase conformation from that of the substrate ATP (26). We found that other competitive inhibitors of ATP binding such as Ap[CH2]3P and H-89 (a protein kinase A inhibitor) do not exhibit similar selectivity as THFA among the adenylyl cyclase isoforms (Figs. 3 and 4), suggesting that inhibiting ATP binding to adenylyl cyclase per se does not produce isoform selectivity. Kinetic analysis of THFA-mediated inhibition of type V adenylyl cyclase demonstrated that the inhibition was not competitive with respect to ATP (Fig. 4C) as expected. It has also been reported that the IC50 values of P-site ligands differ in the presence of various stimulators (25, 27). Forskolin stimulation leads to increased sensitivity to inhibition by 2′-d-3′-AMP with type VI but decreased sensitivity with types I and II, and forskolin decreases the sensitivity of brain adenylyl cyclase to inhibition by 2′,5′-dideoxyadenosine (42). We also examined the IC50 values of THFA when either forskolin or Gsα-5′-GTPγS was used as the stimulator (Figs. 4, A and B) and noted only a small shift to the right in the concentration-response curve.

2-Amino-7-(2-furanyl)-7,8-dihydroxy-5(6H)-quinoxaline and NKY80, a novel type V inhibitor, contains a sub-structure partially similar to adenine (C2H2-N1=C6(NH)2) that we believe plays a key role in inhibiting type V. To our knowledge, this is the first non-nucleoside inhibitor that exhibits isoform selectivity. Although we did not examine its effect on other enzymes such as protein kinases or its effect in intact cells, this compound provides a good start for the synthesis of a chemical series that would enable isoform-selective adenylyl cyclase inhibition.

In conclusion, our findings suggest the feasibility of developing isoform-targeted, therefore tissue-targeted, adenylyl cyclase stimulators and inhibitors by combining computer-assisted drug design algorithms with the findings of recent crystallographic studies.

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