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Protein Engineering of Protein Kinase A Catalytic Subunits Results in the Acquisition of Novel Inhibitor Sensitivity*

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Analysis of the role of specific protein kinases in signal transduction networks has relied heavily on ATP analog inhibitors. Currently used agents, however, often do not distinguish between kinase family members. Genetic approaches can also be used to inactivate a specific kinase, but these techniques do not afford the rapid kinetics possible with pharmacological inhibitors. To circumvent this problem, modification of the structure of a particular protein kinase can be performed to engineer a drug-target interaction of choice. We have used this method to create protein kinase A (PKA) catalytic subunits with modifications that confer sensitivity to novel ATP analog inhibitors. Mutation of methionine 120 to alanine or glycine in either the Cα or Cβ subunits of PKA induces sensitivity to a series of C-3 derivatized pyrazolo[3,4-*d*]pyrimidine-based inhibitors. Modification of threonine 183 enhances this inhibitor sensitivity. The IC_{50} values in cell culture of the most broadly effective agent, 1-NM, ranged from 25 to 200 nM depending upon the combination of modified amino acids and were significantly higher than the potencies observed with H-89. Despite their high sequence conservation, Cβ enzymes with inhibitor-sensitive amino acids at position 120 showed a substantial loss of overall catalytic activity when used to induce reporter gene transcription in transfected cells. Conversion of position 46 (lysine to isoleucine) rescued the ability of position 120 mutated Cβ enzymes to induce gene transcription. Application of this combined genetic and pharmacological approach should allow analysis of the specific roles of PKA isoforms in cell culture and *in vivo*.

Treatment of a physiological system with an inhibitor or activator that is specific for one cellular molecule represents an ideal pharmacological tool. In the case of the protein kinase superfamily, the availability of agents with a specificity for a particular kinase has greatly enhanced our ability to identify the molecular substrates and physiological functions of a given enzyme. Due to the conservation of the catalytic cores of protein kinases, however, the identification of completely specific drugs has remained elusive, and many of agents proposed to be specific have been shown to have multiple targets (1). For example, the commonly used protein kinase A (PKA)[1] inhibitor H-89 effectively inhibits mitogen- and stress-activated protein kinase 1, p70 ribosomal protein S6 kinase, and Rho-dependent protein kinase in addition to PKA (1).

One method that has been employed to circumvent this problem relies upon simultaneous manipulation of the drug and the target to engineer an interaction of choice, an approach termed "chemical genetics" (2). This strategy has been successfully used to examine the function of a number of protein kinases, including v-Src (3–5), Cdc28 (6), and Cla4p (7). The approach takes advantage of the observation that eukaryotic protein kinases have a conserved ATP binding pocket with methionine, isoleucine, threonine, phenylalanine, or leucine at a key position termed the "molecular gate" (8). By mutating this conserved, bulky, hydrophobic residue to a smaller amino acid, mutant kinases have been engineered to accept either inhibitors or phosphodonors that cannot interact with wild-type counterparts (reviewed in Refs. 9 and 10). The ability to selectively inhibit one protein kinase within a cell represents a major advance in the analysis of signal transduction pathways.

PKA is a ubiquitous cellular kinase that phosphorylates serine and threonine residues in response to cAMP. Structurally and biochemically, PKA remains one of the best characterized members of the protein kinase superfamily. The widespread expression of PKA subunits, coupled with the myriad of mechanisms by which cAMP is regulated within a cell, suggests that the PKA signaling cascade is one of general importance to cellular function.

The PKA holoenzyme is composed of two catalytic (C) and two regulatory (R) subunits. In the absence of cAMP, the tetrameric holoenzyme is inactive; when cAMP levels rise, occupation of two cAMP binding sites on each R subunit results in the release of the active C subunits. In mice, there are two C subunit genes encoding the Cα and Cβ isoforms, and differential promoter selection leads to the synthesis of at least two Cα (Cα1 and Cα2 (11, 12)) and three Cβ (Cβ1, Cβ2, and Cβ3 (13)) proteins. The Cα and Cβ subunits exhibit distinct expression patterns, and genetic experiments designed to eliminate each subunit individually have revealed incomplete compensation between the two proteins (14–16). This suggests that there may be specific targets that can only be modified by one of the two forms of the enzyme. To date, the unique roles of Cα and Cβ in the acute transmission of cellular signals have not been fully explored.

We have engineered mutations into the ATP binding pockets of both the Cα and Cβ proteins that confer sensitivity to a series of specific inhibitors that do not inhibit wild-type PKA.

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[1] The abbreviations used are: PKA, cAMP-dependent protein kinase; CREB, cAMP-response element-binding protein; C, catalytic; R, regulatory.

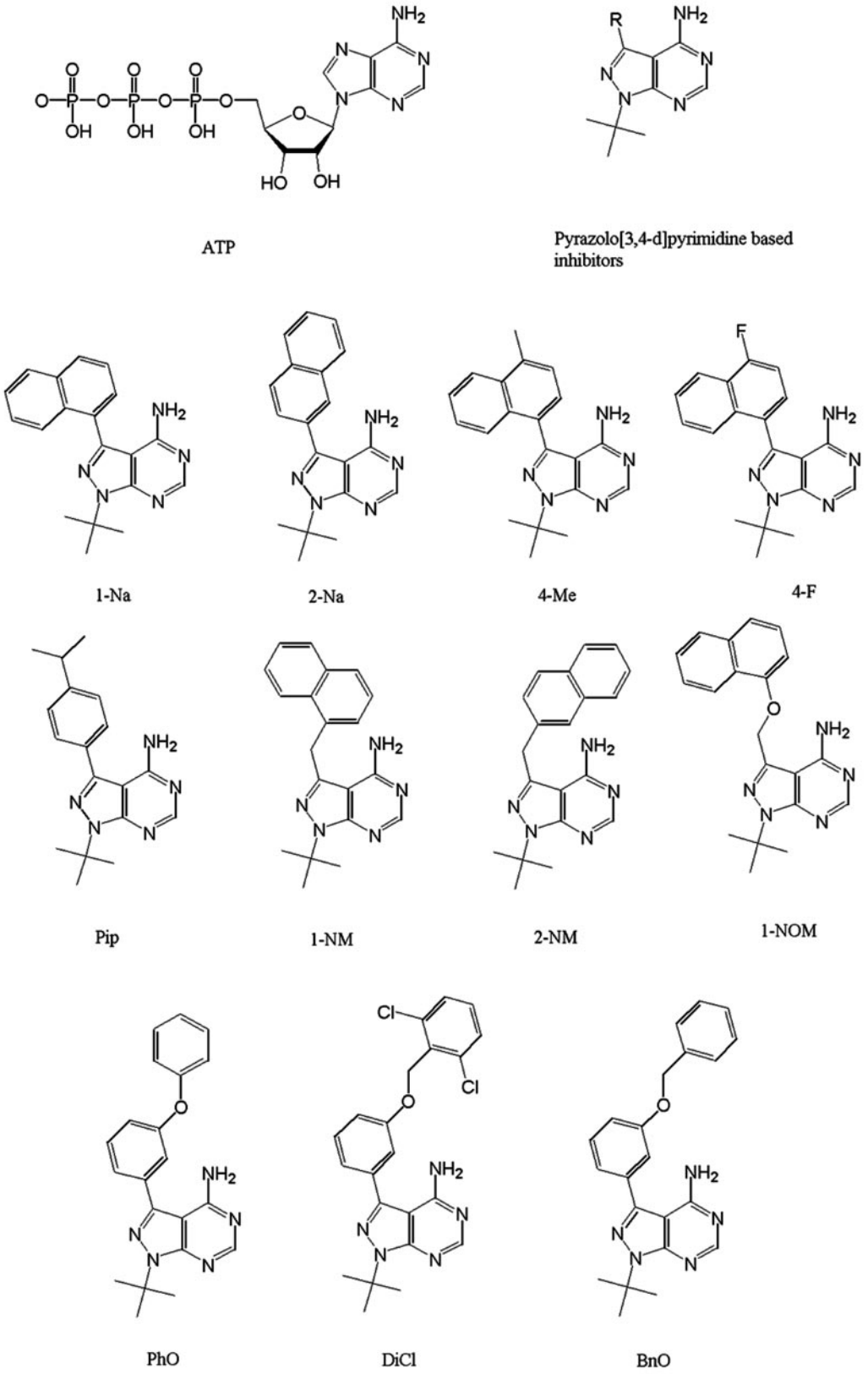

FIG. 1. **Structures of C-3 substituted inhibitors.** The structure of ATP and the core structure of the inhibitors used in this study are shown. *R* indicates the position of modification and the structure of the various derivatives rationally designed according to previous studies with various tyrosine kinases (6, 9).

Interestingly, despite the high degree of sequence conservation, the two subunits exhibit differences in their tolerance of these mutations, suggesting previously unrecognized distinctions in the structure of the Cα and Cβ proteins. Several of the inhibitors blocked the biological activity of the modified PKA C subunits with potencies substantially greater than H-89, the most commonly used ATP analog-based inhibitor of PKA. These results have identified specific mutations in the catalytic

subunits of PKA that produce inhibitor sensitivity without altering biological activity. *In vivo* application of this technology could help delineate physiological functions that specifically require PKA activation as opposed to a related kinase and, in addition, could allow better definition of the specific roles of the Cα and Cβ isoforms.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Previously described plasmid DNAs encoding the mouse Cα and Cβ1 catalytic subunit cDNAs under control of the metallothionein-1 promoter (MT-CEVα and MT-CEVβ (17)) were used as templates for mutagenesis. Mutations were introduced using the QuikChange® mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. Clones were sequenced throughout the entire coding region using the BigDye® sequencing protocol (PerkinElmer Life Sciences) to ensure integrity of the cDNA sequence prior to transfection. Several independent clones were identified and analyzed for the Cβ series of experiments to ensure that results were not influenced by errors in the nonsequenced regions of the vectors.

Cell Culture and Transfection—Human JEG-3 choriocarcinoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone Laboratories), 100 units/ml penicillin, and 100 μg/ml streptomycin, 100 μM nonessential amino acids, and 1 mM sodium pyruvate (Invitrogen). Cells were trypsinized and plated at a density of 1.8×10^5 cells/well in a 24-well plate 3 days prior to transfection. Cells were transfected using calcium phosphate precipitation. Briefly, 2.5 ng of a cAMP-responsive reporter gene (α-168 luciferase) and 50 ng of the internal control plasmid Rous sarcoma virus β-galactosidase and a carrier plasmid (pBluescript KS+; added to a total amount of 250 ng of DNA/well) were transfected in triplicate in the presence or absence of 2.5 ng of the zinc-inducible wild-type or mutant C vectors. Cells were incubated with precipitates for 20–24 h at 37 °C in a 3% CO_2 incubator. Medium was then replaced with the standard medium described above. Approximately 6 h later, cells were treated with medium containing 2.5% fetal bovine serum and 100 μM $ZnSO_4$ in the presence or absence of indicated concentrations of the inhibitor. 18 h later, cells were freeze-thawed and assayed for luciferase and β-galactosidase activity as described previously (18–20). Units of luciferase activity were normalized for transfection efficiency by dividing by β-galactosidase activity.

Molecular Modeling—The sequence of wild-type Cβ1 (Met-120) was submitted to the SwissModel program and folded according to the crystal coordinates of the Protein Data Bank file 1ATP, which represents the crystal structure of Cα complexed in the presence of MgATP and the protein kinase inhibitor (21).

RESULTS

Previous studies have identified a conserved hydrophobic amino acid within the ATP binding site of protein kinases (reviewed in Ref. 10) that functions to define the proper size of the binding pocket. Within the PKA subunits Cα and Cβ, this position corresponds to a methionine residue at position 120. When the analogous site within other protein kinases is changed to alanine or glycine, the resulting kinase will accept substituted ATP analogs or inhibitors that cannot bind to the wild-type enzyme (10). For this study, we employed pyrazolo[3,4-*d*]pyrimidine-based compounds derivatized at the C-3 position of the base ring (Fig. 1). These inhibitors are derived from the core structure of the pyrazolo[3,4-*d*]pyrimidine PP1, a previously described inhibitor of the Src family of protein kinases (22). Bulky substitutions at the C-3 position are predicted to present a hydrophobic surface within the ATP binding pocket that can only be accommodated by a small side-chain amino acid at position 120. In addition, position 183 (a threonine in both Cα and Cβ) is predicted to further constrain the ATP binding pocket and potentially affect binding of the C-3 substituted inhibitors. For this reason, constructs with double modifications at the 120 and 183 positions were also examined for inhibitor sensitivity.

To test the ability of this panel of PP1 derivatives to inhibit the modified enzymes, we chose a transient transfection system using a CREB-responsive luciferase reporter gene as a measure

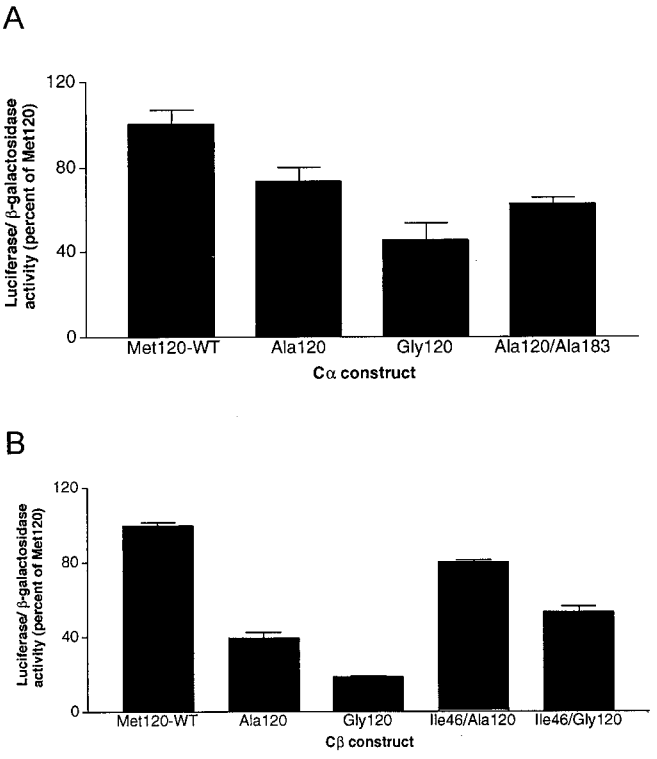

FIG. 2. **Relative efficacy of wild-type and substituted Cα and Cβ enzymes in the induction of CREB-dependent transcription.** Plasmids encoding the indicated Cα1 isoforms (*panel A*, CαMet120, CαAla120, CαGly120, and CαAla120/Ala183) or Cβ1 isoforms (*panel B*, CβMet-120 (wild-type enzyme), CβAla120, CβGly120, CβIle46/Ala120, and CβIle46/Gly120) were transfected in triplicate, and luciferase activity was measured and normalized for transfection efficiency as described under "Experimental Procedures." The activity of the wild-type Met-120 enzymes was set to 100%. Data shown are representative of two to five independent DNA clones for each isoform.

of PKA activity. In this paradigm, cDNAs encoding modified catalytic proteins are overexpressed upon a background level of wild-type kinase, resulting in the expression of mutant catalytic subunits in excess of cellular regulatory proteins. Although a portion of these exogenous subunits will eventually partition into the regulatory subunit pool and displace endogenous wild-type catalytic proteins, high levels of basal kinase activity can be elicited with a great bias toward the transfected catalytic subunits. Drug-induced inhibition of this basal activity can then be assessed with only background levels of contribution from the endogenous enzyme.

Cα and Cβ Differ in Their Tolerance for Modifications at Position 120—Initial transfection experiments using the Cα subunit of PKA revealed a slight loss of catalytic activity when position 120 was converted to an alanine; modification to a glycine resulted in an approximately 50% loss of activity (Fig. 2*A*). Simultaneous substitutions at both the 120 and 183 positions generated enzymes with ~60% catalytic activity as compared with wild type in the absence of added drugs (Fig. 2*A*, Ala-120/Ala-183 enzyme shown). Introduction of either the alanine or glycine 120 substitutions into the Cβ enzyme, however, resulted in a much more pronounced loss of activity as compared with the analogous Cα proteins, particularly when amino acid 120 was modified to a glycine (Fig. 2*B*). Due to the remarkably high level of conservation between the Cα and Cβ proteins, the difference in the ability of the two subunits to tolerate these modifications was unexpected. We isolated several independent clones of the Cβ alanine and glycine 120 constructs to ensure the integrity of the entire plasmid vector used in these studies, and we obtained reproducible levels of

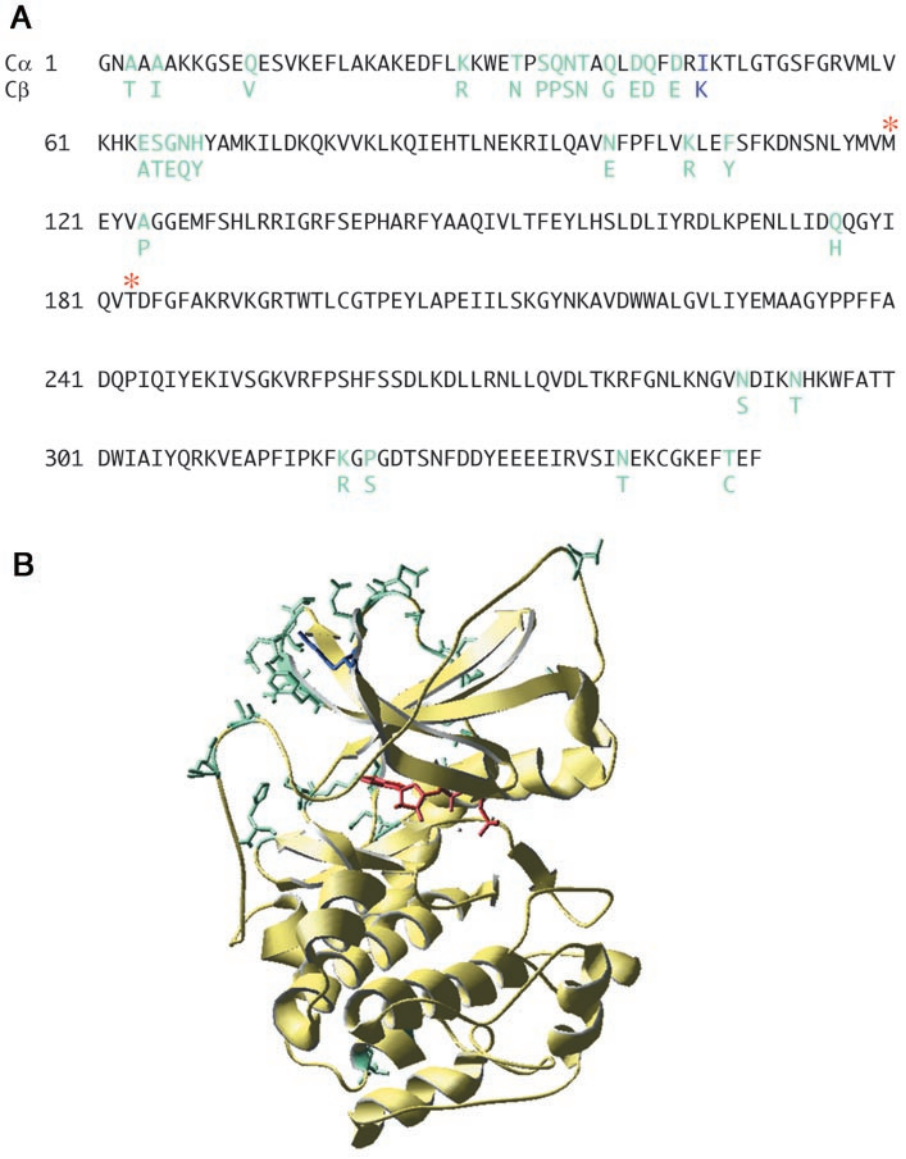

FIG. 3. **Sequence differences between Cα and Cβ and the predicted structure of Cβ.** *A*, a sequence comparison of the mouse Cα and Cβ PKA enzymes. Amino acids that differ between the two proteins are shown in *green*; lysine/isoleucine 46 is highlighted in *blue*. Methionine 120 and threonine 183 are indicated by *red asterisks*. *B*, the structure of Cβ modeled using the crystal coordinates of Cα. Amino acids that differ between the two proteins are shown in *green* with position 46 highlighted in *blue*. ATP is indicated in *red*.

activity when the substitutions were present (data not shown). Comparison of the amino acid sequence of the Cα and Cβ proteins indicates that the majority of differences between the two proteins are conservative substitutions located predominantly in the N-terminal regions of the enzymes (Fig. 3*A*). The exception to these conservative differences is an isoleucine/lysine discrepancy at position 46. Folding of the Cβ sequence using the coordinates of the Cα crystal structure revealed that this amino acid is predicted to reside within the upper lobe of the ATP binding pocket (Fig. 3*B*).

Rescue of Cβ 120-substituted Enzymes by Replacement of Lysine 46 with Isoleucine—In an attempt to rescue the defective kinase activity of the modified Cβ proteins, we substituted the lysine residue present within Cβ with the isoleucine of Cα in the context of the alanine and glycine substitutions (Fig. 2*B*). Transfection analyses of several independent clones revealed a significant rescue of Cβ 120 mutant activity when position 46 of Cβ was also changed to the isoleucine characteristic of Cα. This was particularly evident for the Gly-120 mutant, which exhibited a 5-fold enhancement of catalytic activity in the presence of this isoleucine moiety.

Position 120-substituted Cα and Cβ Variants, but Not Wild-type Enzymes, Are Sensitive to Novel Inhibitors—The sensitivity of transfected Cα and Cβ proteins to the series of C-3 substituted pyrazolopyrimidine inhibitors diagrammed in Fig. 1 was subsequently examined. Table I depicts the ability of a 1 μM concentration of each inhibitor to prevent CREB-dependent transcription in JEG-3 cells transfected with substituted Cα or Cβ subunits. As expected, none of the drugs examined were capable of inhibiting transcription induced by the wild-type (Met-120) enzymes. Several drugs (Pho, DiCl, and BnZO) showed no ability to inhibit any of the modified enzymes at the 1 μM concentration tested; higher amounts of drug also failed to elicit reproducible inhibition (data not shown). A number of agents, however, showed substantial efficacy in blocking the CREB-dependent transcription generated by either the modified Cα or Cβ subunits. The drug sensitivity profiles for mutant Cα and Cβ isoforms were very similar, indicating conserved ATP binding pocket structure between the two proteins. The Cα enzymes containing substitutions at both the 120 and 183 positions, which are hypothesized to have an even larger binding pocket than those modified at position 120 alone, were more sensitive to inhibitors than the singly modified enzymes. Several of the drugs showed a preference for subunits containing either an alanine or a glycine at position 120. For example, the drugs 4-Me and 4-F were more effective at inhibiting transcription from enzymes with alanine at position 120. In contrast, 2-NM and 1-NOM showed a preference for subunits with a

TABLE I

Percent luciferase activity remaining in the presence of C-3 substituted inhibitors and H-89

24 h after transfection, cells were treated with 1 μM or 10 μM (1-NM only) of the C-3 substituted inhibitors or 10 μM of H-89 for 16–20 h, and luciferase activity was subsequently measured. These data are representative of two to six independent determinations performed in triplicate. Data for a given isoform have been normalized to the amount of activity seen in the absence of 1-NM for that enzyme in a particular experiment. Data from the CβGly120 enzyme are omitted from this table as the low activity of this enzyme precluded consistent analysis.

	Cα isoforms						Cβ isoforms			
	Wild-type Met-120	Ala-120	Gly-120	Ala-120/ Ala-183	Ala-120/ Gly-183	Gly-120/ Ala-183	Wild-type Met-120	Ala-120	Ile-46/ Ala-120	Ile-46/ Gly-120
Drug										
1-Na	97.8	24.5	35.4	5.0	4.5	9.1	105.2	47.2	38.3	50.5
2-Na	110.3	46.9	42.6	9.6	7.9	14.2	120.2	51.6	31.6	36.4
4-Me	92.0	21.1	31.8	4.6	6.0	12.6	106.1	28.0	21.5	19.7
4-F	88.4	37.6	54.0	8.2	8.1	16.7	108.6	71.3	66.0	60.2
Pip	98.3	29.7	52.3	11.3	15.2	23.7	98.7	38.3	27.7	54.4
1-NM	96.6	13.1	15.9	7.5	7.0	7.9	118.8	13.5	8.7	10.2
1-NM 10 μM	98.4	2.8	1.8	2.0	2.1	2.1	ND[a]	4.2	4.6	3.7
2-NM	127.4	87.7	51.6	86.9	60.5	30.6	134.2	91.3	91.0	28.9
1-NOM	105.3	88.4	59.6	43.7	45.7	25.3	129.0	83.6	86.7	61.2
Pho	96.6	104.3	90.3	80.0	82.2	96.8	101.1	99.8	92.8	92.7
DiCl	95.5	104.8	97.1	98.2	84.8	100.1	123.5	101.3	100.5	94.6
BnZO	94.4	85.4	121.2	77.3	65.3	88.1	116.8	91.2	97.9	92.9
H-89 10 μM	27.3	49.7	47.0		18.3			47.2	57.4	

[a] ND, not determined.

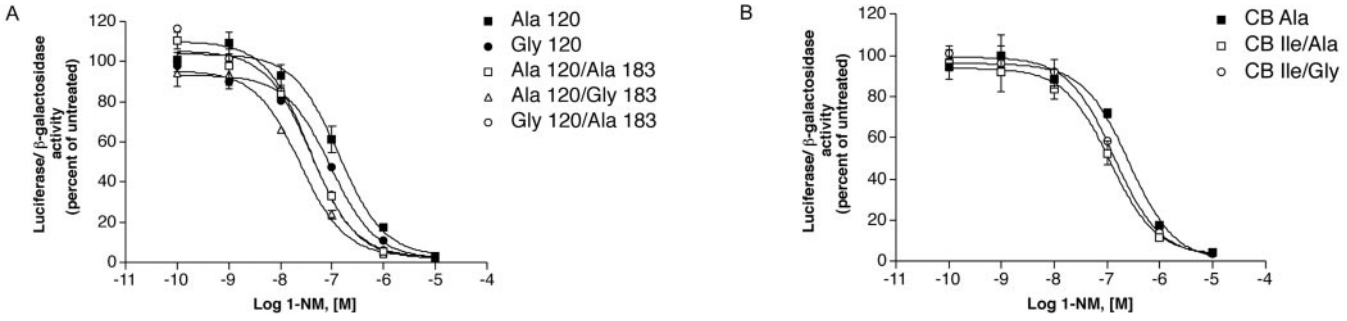

FIG. 4. **1-NM mediates dose-dependent inhibition of CREB-dependent transcription induced by modified Cα (*A*) and Cβ (*B*) isoforms.** Increasing doses of 1-NM were applied to JEG-3 cells transfected with various Cα (*A*) and Cβ (*B*) subunits, and luciferase activity was measured as described under "Experimental Procedures." Data were normalized to the amount of activity measured in the absence of 1-NM and are representative of three to four independent experiments performed in triplicate.

glycine at this position. The most broadly effective inhibitor was the drug 1-NM, which contains a napthylmethyl group at the C-3 position of the pyrazolopyrimidine base ring. This agent effectively reduced the transcriptional activity of all modified Cα and Cβ proteins to background levels without affecting wild-type (Met-120) activity (Table I). Effective drugs were also much more potent than the most commonly used PKA inhibitor, H-89, which only inhibited transcription by approximately ~50% at a 10 μM concentration (Table I). H-89 was, however, a more potent inhibitor of the doubly modified CαAla120/Gly183 as compared with its activity in inhibiting transcription induced by either CαAla120 or CαGly120, similar to the results obtained for many of the C-3 modified pyrazolopyrimidine drugs.

The Agent 1-NM Is a Potent Inhibitor of All Substituted Cα and Cβ Enzymes—Although the transfection method used here does not allow a determination of the exact K_i values for these agents, analysis of the potency of the most globally effective inhibitor, 1-NM, revealed IC_{50} values of 100–200 nM for the CαAla120 and CαGly120 substitutions (Fig. 4*A*). 1-NM was ~2–5 times more potent when interacting with Cα enzymes containing simultaneous alterations at the 120 and 183 positions as compared with subunits modified only at residue 120 (Fig. 4*A* and Table I). Similar IC_{50} values were obtained for the alanine derivatives of both Cα and Cβ (~200 nM), regardless of the isoleucine 46 substitution within Cβ (Fig. 4*B*). The close agreement in 1-NM potency between different versions of Cα and Cβ, both in the presence and the absence of the compensatory position 46 modification, suggests similarities in the way in which the ATP binding pockets of both enzymes interact with these inhibitors.

1-NM Does Not Inhibit Other Components of the cAMP Signaling Cascade in JEG-3 Cells—Although we had induced drug selectivity for PKA enzymes containing alanine or glycine at position 120 over those containing a methionine, it remained possible that these drugs could also inhibit other components of the PKA signaling network such as the ATP-binding protein adenylyl cyclase. To test this, we stimulated wild-type cells containing only endogenous PKA with forskolin in the presence or absence of 1-NM. No significant inhibition of the activity of cellular components leading to CREB-dependent transcription was observed (Fig. 5). Although this experiment cannot rule out the possible interaction of 1-NM with other proteins that bind ATP, it provides evidence that major components of the PKA signaling pathway are not affected by the presence of this agent.

DISCUSSION

The chemical genetic approach represents a novel tool for the study of cellular signal transduction. Due to the similarity between the ATP binding sites of protein kinases, the ability to selectively inhibit one kinase amid other cellular proteins is anticipated to speed the identification of specific phosphorylation targets. This engineered drug-target interaction strategy has been successfully employed to study the function of a number of protein kinases, including Src (3–5) and Cdc28 (6). We

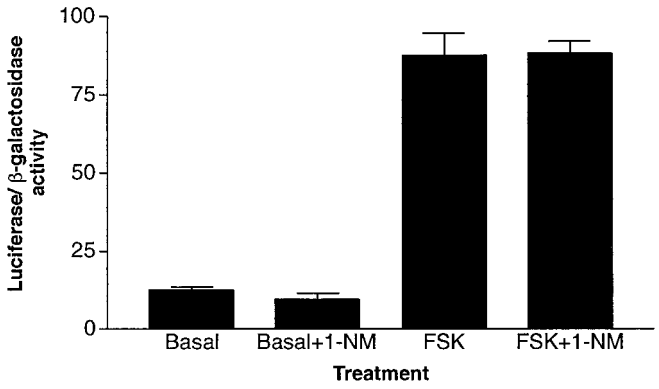

FIG. 5. **1-NM does not inhibit luciferase activity in JEG-3 cells in the absence of transfected modified PKA subunits.** Cells were transfected only with luciferase and β-galactosidase reporter constructs. 24 h later, cells were treated ±forskolin (*FSK*) in the presence of 1 μM 1-NM or vehicle, and luciferase activity was measured after 16 h. These data are representative of three independent experiments (four to six individual wells per experiment).

have now used this method to examine signal transduction induced by the PKA family of enzymes. Substitution within the ATP binding pocket of both the Cα and Cβ enzymes allows for interaction with ATP analogs that normally are unable to compete for ATP binding. The inhibitors chosen for these studies, based upon the structure of the Src inhibitor PP1, contain bulky substituents at the C-3 position that restrict access to the ATP binding pocket of wild-type kinases.

The first criterion inherent to the success of this strategy is that modifications that induce drug sensitivity must have little or no effect on overall activity of the protein in the absence of drug. Modification of methionine 120 to alanine was well tolerated within the Cα protein with levels of activity ~80% of wild type (Fig. 2*A*). Modification to a glycine resulted in lower levels of activity and simultaneous modification at the 120 and 183 positions generated enzymes with 50–60% overall activity (Fig. 2*A*). The analogous mutations in Cβ resulted in much more profound decreases in activity (Fig. 2*B*). It is likely that the alanine and, particularly, the glycine substitutions result in an instability of the Cβ protein similar to that seen when a glycine modification is introduced into the structure of c-Abl (23). For Cβ, rescue of catalytic activity was achieved by replacing the only nonconservative discrepancy between the Cα and Cβ proteins (Lys-46) with the isoleucine characteristic of Cα. Modeling of the Cβ sequence using the coordinates for the Cα crystal structure revealed that this amino acid is predicted to reside within the upper lobe of the ATP binding pocket (Fig. 3*B*).

To date, there are limited studies describing potential differences between the Cα and Cβ proteins. Both of the enzyme variants used in these studies, Cα1 and Cβ1, are expressed ubiquitously. *In vitro* studies have shown that although the two proteins exhibit similar K_m and V_{max} values for ATP, subtle differences are present in substrate selectivity and inhibition by both the protein kinase inhibitor and type II regulatory subunits (24). These results suggest potential distinctions in the physiological functions of Cα and Cβ.

Differences between the PKA catalytic subunits are also apparent from mouse knockout studies. Elimination of the Cβ1 isoform results in healthy animals that display specific defects in hippocampal synaptic plasticity (14); elimination of all Cβ isoforms generates animals with defects in cued fear conditioning (16). Loss of Cα generates an animal that is severely growth retarded with extreme deficits in kinase activity (15). These results indicate that, although some compensation may occur when one subunit is deleted, this compensation is incomplete. These *in vivo* studies, combined with the *in vitro* experiments described above, indicate that important differences between these two highly similar proteins exist. Our results further suggest that there are structural distinctions between Cα and Cβ that revolve around the identity of the amino acid at position 46; this difference may or may not have functional consequences for the activity of the two proteins.

The second critical component of the chemical genetics strategy is the induction of drug specificity only within the modified proteins and not their wild-type counterparts. In these studies, we observed no inhibition of CREB-dependent signal transduction when this series of inhibitors was used in conjunction with the wild-type Cα or Cβ enzymes. In contrast, several drugs were effective at blocking transcription induced by the substituted proteins with similar profiles for Cα and Cβ enzymes. In general, the majority of drugs exhibited a higher degree of inhibition when interacting with proteins substituted at both the 120 and 183 positions. In addition, some drugs inhibited the alanine mutants more effectively than the glycine isoforms (4-Me and 4-F), whereas others showed preferential interaction with the glycine-substituted enzymes (2-NM and 1-NOM). These results suggest that classes of these inhibitors bind uniquely within the ATP binding pocket and that simultaneous modification of two ATP binding site amino acids generally permits enhanced access to this critical portion of the enzyme. Of all inhibitors tested, the agent 1-NM most effectively inhibited all modified enzymes without affecting wild-type activity, even at a 10 μM concentration. Dose-response analyses of this agent revealed an IC_{50} range of ~25–200 nM with increased potencies at the enzymes modified at both the 120 and 183 positions. It should be noted that these potencies were observed in the presence of normal cellular concentrations of ATP, predicted to be in the millimolar range. The potency of 1-NM was also manyfold higher than that observed using H-89, one of the most commonly used PKA inhibitors.

In conclusion, we have shown the viability of the chemical genetics strategy for the study of PKA-mediated signaling *in vivo*. We have produced several modifications within the Cα and Cβ enzymes that confer inhibitor sensitivity while retaining near wild-type levels of catalytic activity. Use of the agent 1-NM provides a potent and selective means of inhibiting only modified PKA isoforms with no observed effect on other components of the PKA pathway (Fig. 5). Introduction of these modifications into the Cα and Cβ genes using embryonic stem cell technology will allow the study of acutely regulated PKA function in a whole mouse or in tissues and cells derived from such a modified animal. It should be noted that both Cα and Cβ heterozygotes are phenotypically normal (13, 15), suggesting that a slight reduction in overall kinase activity in the absence of drugs (such as that seen with the CαAla120 isoform) will not prove detrimental to the animal. The approach applied here, designed to engineer a drug-target interaction of choice, should prove invaluable in the study of the role of specific protein kinases in signal transduction.

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