

# Inhibition of Protein Kinases by Balanol: Specificity within the Serine/Threonine Protein Kinase Subfamily

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## ABSTRACT

Balanol is a potent inhibitor of cyclic AMP-dependent protein kinase and protein kinase C, acting competitively with ATP with an affinity 3000 times that of ATP. We tested the capacity of balanol to inhibit representative serine- and threonine-specific protein kinases from the protein kinase subfamily that shares a common conserved catalytic core with cyclic AMP-dependent protein kinase. Balanol's pattern of interactions indicates con-

siderable diversity of the ATP/balanol-binding sites of protein kinases within familial groups and even among isoforms of the same kinase. We propose that balanol is a protean structure that may be modified to produce selective, high-affinity inhibitors and probes of the ATP-binding sites of serine/threonine protein kinases.

Balanol, a natural product of the fungus *Verticillium balanoides* (Kulanthaivel et al., 1993), has also been synthesized chemically (Nicolaou et al., 1994). The molecule consists of three regions: benzophenone, hexahydroazepane, and 4-hydroxy benzoyl moieties. The benzophenone and hexahydroazepane moieties are connected through an ester linkage; the azepane and 4-hydroxy-benzoyl moieties are interconnected by an amide linkage (Fig. 1). Balanol is a potent inhibitor of cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC), kinetically appearing to interact competitively with ATP on the catalytic domains of PKC and PKA (Koide et al., 1995). In fact, balanol interacts with these protein kinases with an affinity ( $K_i \geq 4$  nM) that is more than three orders of magnitude greater than that for ATP. Balanol does not inhibit two tyrosine protein kinases, the Src kinase and the epidermal growth factor receptor kinase (Koide et al., 1995).

We recently described the crystal structure of the complex formed by the catalytic subunit of PKA and balanol (Narayana et al., 1999). The structural features of that report confirm the prediction of the kinetic data: balanol is an ATP congener and inhibits kinase activity by binding in the ATP-binding pocket. Furthermore, identifiable regions of balanol correspond to the adenine ring, ribose, and phosphate groups

of ATP, and modifications of balanol, especially in rings C and D that are the phosphate analogs, produce inhibitors of protein kinases that have unusual potency and specificity.

On the basis of these data, we hypothesized that balanol would inhibit all serine/threonine protein kinases that share a conserved ATP-binding site structure within the kinase common catalytic core. To test this hypothesis, we assessed the capacity of balanol to inhibit representatives of several groups within the serine/threonine protein kinase subfamily (Hardie and Hanks, 1995). Specifically, we tested balanol against members of the second messenger-regulated protein kinases that are classified as "basic amino acid-directed enzymes" [the AGC group: PKA, cyclic GMP (cGMP)-dependent protein kinase (PKG), PKC, including isoforms of PKC and PKA with specific mutations]; against representatives of the  $\text{Ca}^{2+}$ -calmodulin-regulated kinases [the calmodulin-activated kinase (CaMK) group, represented by smooth muscle myosin light chain kinase (smMLCK), phosphorylase kinase (PhK), and CaMKII]; and against several protein kinases of the CMGC group: mitogen-activating protein kinase (MAPK/Erk1), a cyclin-dependent kinase ( $\text{p34}^{\text{cdc2}}$ ), and casein kinase II (CKII), enzymes that prefer serine/threonine residues within a proline-rich environment.

We have also observed that minor modification of the balanol structure produces congeners that exhibit selectivity toward PKA over PKC of two to three orders of magnitude. For instance, 14"-decarboxybalanol inhibits PKA with a  $K_i$  value

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**ABBREVIATIONS:** PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; cGMP, cyclic GMP; PKG, cyclic GMP-dependent protein kinase; PhK, phosphorylase kinase; smMLCK, smooth muscle myosin light chain kinase; CaMKII,  $\text{Ca}^{2+}$ -calmodulin-activated kinase II; MAPK, mitogen-activated protein kinase;  $\text{p34}^{\text{cdc2}}$ , cyclin-dependent kinase; CKII, casein kinase II.

of 11 nM and PKC with a  $K_i$  value of 5000 nM. This is a surprising result because both PKA and PKC are closely related to protein kinases that share a common catalytic core (Hardie and Hanks, 1995; Narayana et al., 1999). These results suggested that balanol might be a useful template on which a family of specific protein kinase inhibitors could be developed. To test this possibility, we determined the inhibitory effects of two PKA-selective congeners of balanol on several protein kinases that balanol, itself, inhibits.

The results indicate that balanol and its congeners are effective inhibitors of some, but not all, of the Ser/Thr protein kinase subfamily that share a common catalytic core. The specificity of balanol toward certain kinases within this subfamily suggests a considerable microdiversity of ATP/balanol-binding regions within familial subgroups of protein kinases and even between isoforms of the same protein kinase.

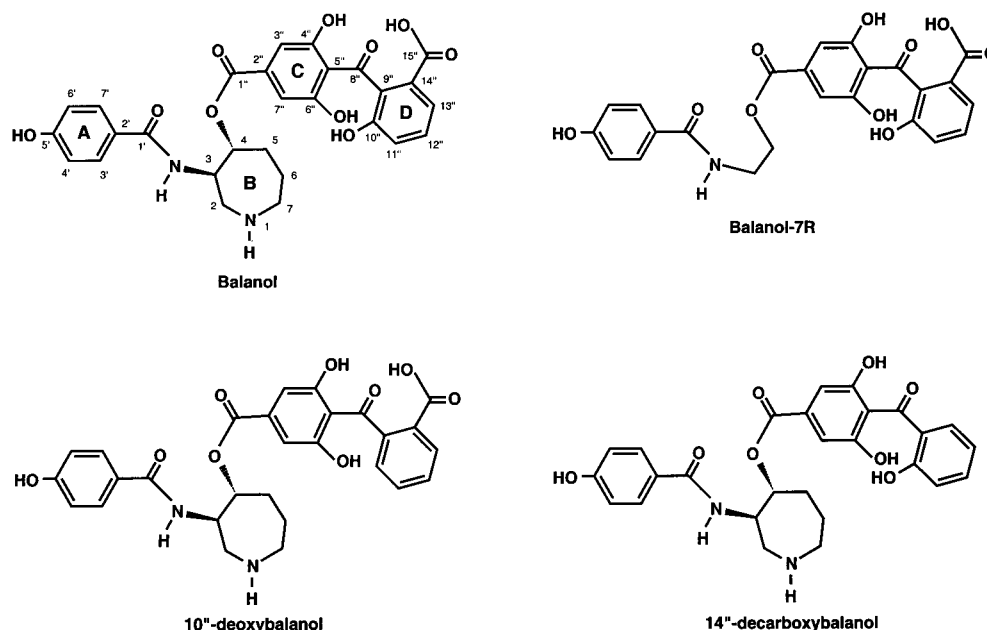
## Experimental Procedures

**Materials.** Balanol and its derivatives were synthesized as described previously (Nicolaou et al., 1994; Koide et al., 1995). Histone H1, PhK, p34<sup>cdc2</sup>, and glycogen phosphorylase were purchased from Upstate Biotechnology (Lake Placid, NY). PKG $\alpha$ , CKII, and CKII substrate were purchased from Promega (Madison, WI). Calmodulin and CaMKII were purchased from Calbiochem (San Diego, CA). PKG substrate was purchased from Peninsula Laboratories (Belmont, CA). Erk1 antibody was purchased from Santa Cruz Biochemicals (Santa Cruz, CA). [ $\gamma$ -<sup>32</sup>P]ATP was purchased from ICN (Costa Mesa, CA). PKC $\alpha$  and PKC $\beta$ II were gifts from Dr. Alexandra Newton (University of California at San Diego, La Jolla, CA). smMLCK was a gift from Dr. Primal de Lanerolle (University of Illinois, Chicago, IL). Glycine-rich loop mutants of PKA (S53G and F54E) were donated by Dr. Susan Taylor (University of California at San Diego, La Jolla, CA). All other reagents and chemicals were reagent grade from Aldrich-Sigma (St. Louis, MO).

**Assay of Protein Kinase Activities.** Assays of protein kinase activities were performed using previously described methods (Koide et al., 1995) in 100- $\mu$ l reactions at 30°C for 10 min and were based on

the transfer of the  $\gamma$  phosphate of [ $\gamma$ -<sup>32</sup>P]ATP to a suitable substrate, under conditions appropriate for the individual enzymes. Activities were linear functions of time and protein over the ranges used. Each experimental condition was duplicated within each assay, and each assay was replicated two to five times, as noted. Protein content was estimated by the method of Bradford (1976) using BSA as a standard.

Activities of PKA and PKC were assessed as previously described (Koide et al., 1995). PKG activity was assessed by the addition of 12.5 ng of the purified enzyme to a reaction mixture consisting of 20 mM Na<sup>+</sup>-HEPES (pH 7.5 at 30°C), 10 mM MgCl<sub>2</sub>, 0.2  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP, 100  $\mu$ M 3-isobutyl-1-methylxanthine, 130  $\mu$ M substrate (R-K-R-S-R-A-E), 30  $\mu$ M ATP, and 30  $\mu$ M cGMP (adapted from Sekhar et al., 1992). PhK activity was assessed by the addition of 300 ng of the purified enzyme to a reaction mixture consisting of 50 mM Tris · HCl (pH 8.2 at 30°C), 10 mM MgCl<sub>2</sub>, 0.1 mg/ml BSA, 50  $\mu$ M ATP, 200  $\mu$ g/ml glycogen phosphorylase, 1 mM  $\beta$ -mercaptoethanol, 1  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP, 500  $\mu$ M CaCl<sub>2</sub>, 50 mM  $\beta$ -glycerolphosphate, and 2.5  $\mu$ M calmodulin. smMLCK activity was assessed as described by Strauss et al. (1995). CaMKII activity was assessed by the addition of 20 ng of purified enzyme to a reaction mixture consisting of 50 mM Na<sup>+</sup>-HEPES (pH 7.5 at 30°C), 10 mM MgCl<sub>2</sub>, 50  $\mu$ M ATP, 1 mg/ml myelin basic protein, 500  $\mu$ M CaCl<sub>2</sub>, 3  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP, and 2.5  $\mu$ M calmodulin. p34<sup>cdc2</sup> activity was assessed by the addition of 10 ng of the purified enzyme to a reaction mixture consisting of 50 mM Na<sup>+</sup>-HEPES (pH 7.2, 30°C), 25 mM  $\beta$ -glycerolphosphate (pH 7.5), 5 mM EGTA, 20 mM MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM dithiothreitol, 0.5 mg/ml histone H1, 30  $\mu$ M ATP, and 2.5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (adapted from Sanghera et al., 1990). CKII activity was assessed in a 20-min assay by adding 6 to 23 ng of purified enzyme to a reaction mixture consisting of 25 mM Na<sup>+</sup>-HEPES (pH 7.5 at 30°C), 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 30  $\mu$ M ATP, 1  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP, 50 mM synthetic substrate (R-R-R-E-E-E-T-E-E-E), 0.1 mg/ml BSA, and 50 mg/ml poly(L-lysine; adapted from Criss et al., 1978). MAPK was isolated from cultured A431 human epidermal carcinoma cells by immunoprecipitation as described by Hilal-Dandan et al. (1997). MAPK activity was assessed by the addition of 200  $\mu$ g of enzyme-antibody complex to a reaction mixture consisting of 30 mM Na<sup>+</sup>-HEPES (pH



**Fig. 1.** Structures of balanol and several derivatives. Balanol consists of a 4-hydroxy benzamide moiety (A) joined by an amide linkage to a hexahydroazepane moiety (B) which is coupled via an ester linkage to a benzophenone (C and D). In terms of analogy to ATP, balanol's ring A and the amide linker correspond to the adenine of ATP, ring B to the region of the ribose, rings C and D to the triphosphates (see Narayana et al., 1999; Gustafsson and Brunton, 1999).

7.5 at 30°C), 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 20 μM ATP, 1 mg/ml myelin basic protein, and 1 μCi of [γ-<sup>32</sup>P]ATP.

**Membrane Preparation and Assay of Adenylyl Cyclase Activity.** Cultured HeLa and A431/29i cells, grown in Dulbecco's modified Eagle's medium with 10% FBS, were serum starved for 30 min and collected by scraping in hypotonic buffer (50 mM β-glycerol phosphate, pH 7.5, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 10 μg/ml leupeptin, 300 μM phenylmethylsulfonyl fluoride, and 100 μM Na<sub>3</sub>VO<sub>4</sub>, at 4°C). The suspension was homogenized, and membranes were collected by centrifugation (22,000g for 20 min). Adenylyl cyclase activities were assessed in the resuspended pellets using the method of Salomon et al. (1974) with 500 μM ATP (the  $K_d$  value for ATP is 250 μM for this enzyme; Dessauer and Gilman, 1997).

**Analysis and Graphing.** Statistical analysis and graphing of data were accomplished with the program InPlot4 (GraphPAD Software, San Diego, CA). The  $K_i$  values for balanol and its derivatives were determined as described previously (Koide et al., 1995) using the equation  $K_i = (IC_{50} * K_d) / (K_d + L)$ , where  $K_i$  is the inhibition constant,  $IC_{50}$  is the concentration of the inhibitor needed to cause 50% inhibition of enzyme activity,  $K_d$  is the apparent dissociation constant of ATP for the protein kinases (determined by an experiment for each enzyme: PKA, 16 μM; PKC isoforms, 20 μM; PKG, 29 μM; p34<sup>cdc2</sup>, 13 μM; CaMKII, 60 μM; MAPK, 16 μM), and  $L$  is the total concentration of ATP used in the assay. Curves shown are representative of two to five replicate experiments, of which the  $K_i$  values were averaged to obtain the values shown in the text.

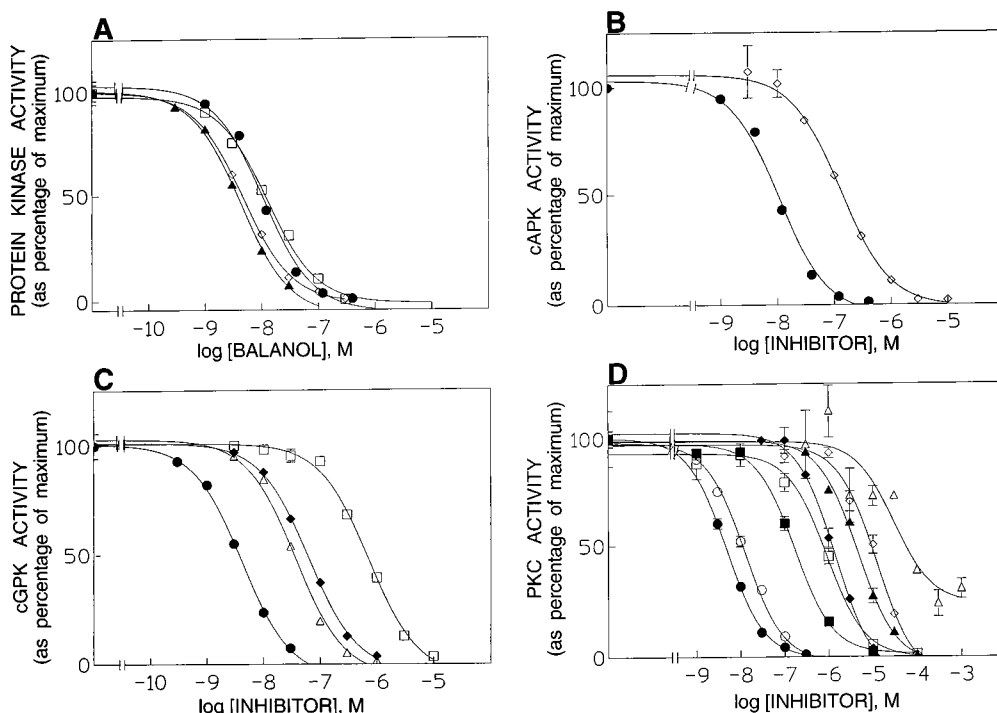
## Results

**General Approach.** Within each group of protein kinases, we first tested balanol as an inhibitor (Koide et al., 1995). In each instance in which balanol inhibited protein kinase activity in these experiments, the effect of balanol was sur-

mounted by increasing concentrations of ATP. This competitive interaction of ATP and balanol provided evidence that balanol interacts at the ATP binding site of the kinase, as demonstrated directly for PKA by analysis of a balanol-PKA catalytic subunit crystal (Narayana et al., 1999). For protein kinases toward which balanol exhibited high inhibitory potency, we examined the effect of balanol derivatives that exhibited specificity for PKA over PKC (e.g., 14"-decarboxybalanol and 10"-deoxybalanol) as a measure of structural diversity among the catalytic cores of the protein kinases.

**Interaction of Balanol with AGC Group.** The inhibitory constant ( $K_i$ ) of balanol toward the catalytic subunit of PKAα is 3.9 nM (derived from data of Fig. 2A), in good agreement with the data of Koide et al. (1995; 4 nM), confirming the experimental and analytical techniques used. The  $K_i$  value of balanol toward PKG is 1.6 ± 0.12 nM (mean ± S.E.;  $n = 5$ ; Fig. 2A). We tested the possibility that balanol might inhibit PKG activity by interacting with cGMP-binding sites on PKGα; this seems unlikely because the effect of balanol (at 3 nM, producing ~60% inhibition of PKG activity) was constant over a wide range of cGMP concentrations (0–300 μM). The  $K_i$  values of balanol toward PKCα and PKCβII are 6.4 ± 0.8 nM (mean ± S.E.;  $n = 3$ ) and 1.8 ± 0.04 nM (mean ± range;  $n = 2$ ), respectively (Fig. 2A).

The similar potencies of balanol toward different members of the AGC group could be interpreted as supporting the concept of a conserved ATP binding site within the common catalytic core of these protein kinases. However, derivatives of balanol (Fig. 1) can distinguish among the kinases. The  $K_i$  value of a balanol derivative lacking the seven-membered



**Fig. 2.** Effects of balanol on the AGC group of protein kinases. A, inhibition of PKAα (●), PKGα (▲), PKCα (□), and PKCβII (◇). The average  $K_i$  values for balanol are PKAα, 3.9 nM; PKGα, 1.6 nM; PKCα, 6.4 nM; and PKCβII, 1.8 nM. B, comparison of effects of balanol (●) and Bal-7R (◇) on PKAα. Data are mean ± range of duplicate samples. Calculated  $K_i$  values are 3.9 nM (balanol) and 47.3 nM (Bal-7R). C, comparative effects of balanol and congeners on PKGα. Calculated  $K_i$  values are 1.8 nM (balanol, ●), 19 nM (14"-decarboxybalanol, △), 30.7 nM (10"-deoxybalanol, ◆), and 291 nM (Bal-7R, □). D, comparative effects of balanol and congeners on PKC isoforms. Open symbols are for PKCα; closed symbols are for PKCβII. Balanol congeners are represented by different symbols: circles represent balanol, squares represent Bal-7R, diamonds represent 10"-deoxybalanol and triangles represent 14"-decarboxybalanol. Calculated  $K_i$  values are given in the text.



ring (Bal-7R) on PKA is  $47.3 \pm 1.1$  nM (mean  $\pm$  S.E.;  $n = 2$ ; Fig. 2B). The  $K_i$  values for 14"-decarboxybalanol and 10"-deoxybalanol on PKA are 11 and 3.9 nM (Koide et al., 1995). Thus, eliminating the seven-membered ring from balanol backbone has a modest effect on balanol's affinity toward the ATP binding site on PKA, whereas omission of the carboxy or hydroxy functional groups from 14" or 10" positions on balanol does not affect the binding affinity toward PKA. The  $K_i$  values for balanol, 14"-decarboxybalanol, and 10"-deoxybalanol toward PKG are  $1.6 \pm 0.1$  (mean  $\pm$  S.E.;  $n = 5$ ),  $19.0 \pm 1.2$  (mean  $\pm$  range;  $n = 2$ ), and  $30.7 \pm 0.4$  nM (mean  $\pm$  range;  $n = 2$ ), whereas the  $K_i$  value for Bal-7R is 291 nM (Fig. 2C), nearly two orders of magnitude greater than that for balanol itself. The effects of these derivatives on PKG are not identical with their effects on PKA: toward PKG, 14"-decarboxylation and 10"-dehydroxylation increase the  $K_i$  by about one order of magnitude, and removal of the seven-membered ring shifts the  $K_i$  by two orders of magnitude.

The  $K_i$  values of Bal-7R toward PKC $\alpha$  and PKC $\beta$ II are 347 and 65.2 nM, respectively (Fig. 2D). The  $K_i$  value of 10"-deoxybalanol is 2910 nM toward PKC $\alpha$  and 550 nM toward PKC $\beta$ II. The  $K_i$  values of 14"-decarboxybalanol for PKC $\alpha$  and PKC $\beta$ II are 11,680 and 2000 nM (Fig. 2D). Thus, elimination of either one of these functional groups greatly affects the affinity of balanol toward both isoforms of PKC. All of the balanol derivatives tested are five to six times more potent toward PKC $\beta$ II than PKC $\alpha$ . In general, alterations in the structure of balanol emphasize the complementary nature of the interactions between balanol and kinases of the AGC group, a complementarity that is distinct for each kinase.

We also investigated the effect of mutations in the glycine-rich loop of the catalytic subunit of PKA on the interactions of balanol and ATP with the enzyme. The F<sup>54</sup>G mutation shows affinities for ATP (15  $\mu$ M) and balanol (8 nM) similar to those for the wild-type enzyme (18  $\mu$ M and 4 nM, respectively). These findings support the idea that the interactions of the backbone atoms of this residue are more important than effects of the side chain of F54. However, the F<sup>54</sup>E mutant shows slightly larger dissociation constants (ATP, 59 nM; balanol, 13 nM), as might be expected from placing a negatively charged group in a position that would interfere with the negatively charged substituent on balanol or with the  $\gamma$  phosphate of ATP. In an S<sup>53</sup>G mutant, we observe negligible effects on the  $K_i$  value for balanol (4 nM) or on the  $K_m$  value for ATP (34  $\mu$ M). These results coincide with the findings discussed in detail in two related reports (Hunenberg et al., 1999; Narayana et al., 1999), that a major determinant of the affinity and specificity of the balanol-kinase interaction is the overall shape complementarity of the ligand to the kinase.

**Interaction of Balanol with CaMK and CMGC Groups.** Compared with its effects on kinases from the AGC group, balanol is a less potent inhibitor of CaMKII ( $K_i = 74.2 \pm 29.5$  nM; mean  $\pm$  range;  $n = 2$ ; see Fig. 3A). Over a wide range of concentrations, balanol does not inhibit PhK or smMLCK activities (Fig. 3A). Thus, relative to its affinity toward protein kinases of the AGC group, balanol is only a modestly potent inhibitor of select members of the CaMK group.

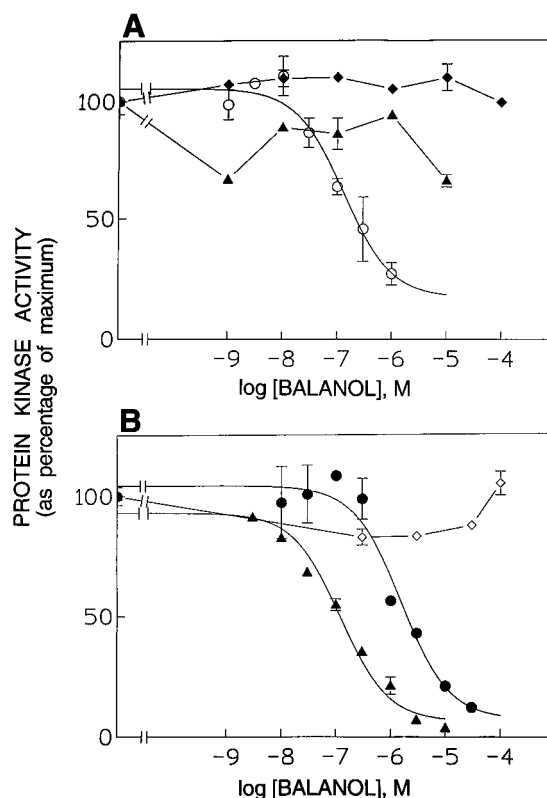
Members of the CMGC group vary widely in their susceptibility to the inhibitory effects of balanol (see Fig. 3B). Balanol inhibits the activity of p34<sup>cdc2</sup> with a  $K_i$  value of  $29.7 \pm$

2.7 nM (mean  $\pm$  S.E.;  $n = 3$ ) and MAPK (Erk1) activity with a  $K_i$  value of  $742 \pm 49$  nM (mean  $\pm$  range;  $n = 2$ ). Concentrations of balanol as high as 100  $\mu$ M do not inhibit CKII.

**Interaction of Balanol with G<sub>s</sub> and Adenylyl Cyclase.** If balanol is to be a pharmacologically useful inhibitor of hormonally regulated protein kinases in intact cells, it is important to know whether balanol interacts with nucleotide triphosphate-binding sites involved in the production of regulatory second messenger (e.g., the GTP recognition site on heterotrimeric G proteins and the ATP-binding site on adenylyl cyclase). We approached this question by assessing the effects of balanol on forskolin- and isoproterenol-stimulated adenylyl cyclase activity. Under conditions where forskolin and isoproterenol produce 2- to 5-fold elevations of adenylyl cyclase activity in membranes of cultured HeLa and A431 cells, balanol (up to 100  $\mu$ M) does not inhibit either basal, forskolin-stimulated, or hormone-stimulated enzyme activities (data not shown; [ATP] = 500  $\mu$ M). Thus, in *in vitro* assays, balanol inhibits PKA but not the cellular signaling system ( $\beta$  receptor-G<sub>s</sub>-adenylyl cyclase) that generates the second messenger that activates PKA. Whether balanol is actually an effective inhibitor of PKA in whole cells is addressed in the accompanying article (Gustafsson and Brunton, 1999).

## Discussion

Balanol is a potent competitive inhibitor of ATP binding to several serine/threonine protein kinases. Analysis of the



**Fig. 3.** Effects of balanol on the CaMK and CMGC groups. A, effects of balanol on the CaMK group. Calculated  $K_i$  value for balanol as an inhibitor of CaMKII ( $\circ$ ) is 74.2 nM. Balanol does not inhibit smMLCK ( $\blacktriangle$ ) or PhK ( $\blacklozenge$ ). Data are mean  $\pm$  range of duplicate samples. B, effects of balanol on the CMGC group. Calculated  $K_i$  values for balanol are 29.7 nM (p34<sup>cdc2</sup>,  $\blacktriangle$ ) and 742 nM (MAPK,  $\bullet$ ). Balanol does not inhibit CKII ( $\diamond$ ). Data are mean  $\pm$  range of duplicate assays.

structure of the balanol-PKA complex indicates that balanol binds in the ATP site of the catalytic cleft of PKA (Narayana et al., 1999). In addition, balanol binds to PKA and PKC with about three orders of magnitude greater affinity than ATP, a feature of balanol that emphasizes its potential use as an inhibitor and as a probe of ATP-binding sites of protein kinases.

Given balanol's homology to ATP and its high affinity toward protein kinases, we expected balanol to inhibit any protein kinase whose catalytic core resembles that of PKA. Recent studies of protein kinase structure have stressed the similarities of the catalytic cores of serine/threonine kinases (Taylor et al., 1992a,b; Zheng et al., 1993; Taylor and Radzio-Andzelm, 1994) and have grouped protein kinases according to sequence, structural homology, and biochemical properties such as modes of regulation, substrate recognition sequence motifs, and substrate specificity (Hanks et al., 1988; Hanks and Quinn, 1991; Hardie and Hanks, 1995). Within the serine/threonine protein kinase family, major subgroups are the AGC, CaMK, and CMGC groups. Despite the apparent structural homologies within the catalytic cores of these groups, balanol does not uniformly inhibit these kinases. Thus, although the apparent affinities of these enzymes for ATP vary over a relatively narrow range (13–60  $\mu$ M), the  $K_i$  values for balanol range very widely, from 1.6 to 742 nM (see Table 1), and several members of the serine/threonine protein kinase family are not inhibited by balanol.

This range of balanol's effectiveness is best illustrated by "normalized" inhibition curves (Fig. 4), generated using experimentally determined affinities for ATP and balanol to calculate theoretical activities of each kinase in a hypothetical assay (see legend to Fig. 4; this extrapolation is necessitated by the need, in real experiments, to use different ATP concentrations for the different enzymes; thus, raw data and  $IC_{50}$  values are not useful comparisons). As Fig. 4 makes clear, balanol is a very potent inhibitor of the AGC group, with  $K_i$  values between 1.6 and 6.4 nM, indicating that balanol binds to these enzymes three to four orders of magnitude more avidly than does ATP. For protein kinases of the CaMK and CMGC subgroups, the effects of balanol vary: generally, balanol is a less potent inhibitor of these kinases and is sometimes without effect. In the CaMK group, the  $K_i$  value of balanol toward CaMKII is 74 nM, and balanol does not inhibit the activity of either PhK or smMLCK. Within the CMGC group, the  $K_i$  values of balanol range from 30 nM for p34<sup>cdc2</sup> to 742 nM for MAPK (Erk1), and balanol does not inhibit the activity of CKII.

From the proposed conservation of a common catalytic core (Taylor et al., 1992a; Hardie and Hanks, 1995; Narayana et al., 1999), we expected balanol to inhibit all of these protein kinases. Evidently, there is more structural dissimilarity among the catalytic cores of the kinases outside the AGC group than anticipated. On the other hand, balanol potently inhibits CaMKII and p34<sup>cdc2</sup>; this suggests that significant

differences exist within the ATP/balanol-binding domains of kinases in the CaMK and CMGC groups. The effectiveness of balanol to inhibit CaMKII, p34<sup>cdc2</sup>, and MAPK suggests that derivatives of the balanol could be designed that would be potent inhibitors of these protein kinases. All in all, the data reflect the conserved structure within the AGC group and suggest that there is less conservation within the ATP/balanol-binding domains of protein kinases in the CaMK and CMGC groups. As noted below, however, a small number of strategic differences, possibly even single amino acid substitutions, can dramatically alter the inhibitor-kinase interaction.

The effects of derivatives of balanol suggest some diversity of the ATP/balanol-binding sites within the AGC subgroup. The elimination of functional groups from ring D of the benzophenone to produce the 14"-decarboxy and 10"-deoxy derivatives (Fig. 1) causes a substantial loss of affinity (roughly three orders of magnitude) toward both the  $\alpha$  and  $\beta$ II isoforms of PKC. However, the 14" decarboxy and 10" deoxy congeners are equipotent with balanol against PKA and only slightly less potent than balanol against PKG $\alpha$ . In general, modifications of ring D of balanol increase the specificity of balanol toward PKA over PKC. Against the AGC group of kinases, the effect of eliminating balanol's azepane ring is a reduction in affinity that is greatest toward PKG (two to three orders of magnitude) and substantially less toward PKC ( $\alpha$  and  $\beta$ II) and PKA.

As discussed more fully in our recent article on the structure of the PKA-balanol complex (Narayana et al., 1999), in PKA, the 14" carboxylate of balanol interacts with E91, located on  $\alpha$ -helix C of the PKA catalytic subunit, and the 10"-hydroxyl group of balanol interacts with S53, located in the glycine-rich loop (Zheng et al., 1993). Neither 14"-decarboxylation nor 10"-dehydroxylation of balanol has a major effect on the affinity toward PKA. This is likely explained by the cancellation of the favorable contribution of hydrogen bonding by the energetic cost of desolvation: thus, the formation of H-bonds makes a negligible contribution to the binding affinity but may contribute to specificity (Hunenburger et al., 1999). However, such reasoning seems unlikely to explain why 14"-decarboxylation or 10"-dehydroxylation of balanol greatly reduces the apparent affinity toward PKC isoforms. These seemingly conflicting results may be explained by the flexibility of balanol (e.g., rotation of ring D; see Fig. 1), flexibility that may permit compensatory interactions to form in PKA but not in PKC due to unique microenvironments. The overall rigidity of the ATP-binding site and of the protein-balanol complex may be important determinants of balanol's specificity. Mendoza et al. (1995) addressed the issue of rigidity of the ligand, substituting conformationally constrained bicyclic and tricyclic rings for the azepane ring (ring B in Fig. 1). These rigidified derivatives are more potent and selective for PKC over PKA, possibly reflecting different flexibilities of the ATP-binding domains of PKC and PKA.

TABLE 1  
Apparent affinities for balanol and ATP

	Protein Kinase						
	PKG	PKC $\beta$ II	PKA	PKC $\alpha$	p34 <sup>cdc2</sup>	CaMKII	MAPK
$K_i$ for balanol, nM	1.6	1.8	3.9	6.4	30	74	742
$K_d$ for ATP, $\mu$ M	29	20	16	20	13	60	16

The relative specificity of the 14"-decarboxyl or 10"-dehydroxyl derivatives for PKA over PKC may offer a clue as to what structural features of protein kinases contribute to the specificity of the balanol-kinase interactions. A single amino acid change can confer selectivity on inhibitor-kinase interactions such as we observe with respect to PKA and PKC and the 14" and 10" derivatives of balanol. For example, there is a pyridinylimidazole compound that inhibits ATP binding to p38 MAPK and thus inhibits the enzyme's activity (Wilson et al., 1997). The inhibition is relatively specific for p38 MAPK over other MAP kinases, a specificity that can be explained by a single amino acid difference: in p38, residue 106 is a threonine that interacts with the adenine ring of ATP and with the *p*-fluorophenyl ring of the pyridinylimidazole inhibitor. In kinases resistant to pyridinylimidazoles, Thr<sup>106</sup> is replaced by methionine (JNK family) or glutamine (ERK1/2; Wilson et al., 1997).

A similar paradigm, applied to the phosphate-binding domain, may account for our observation that the 14"-decarboxyl and 10"-dehydroxyl derivatives of balanol show several orders of magnitude specificity for PKA over PKC. The 14" and 10" positions in balanol interact with regions of the protein kinases that normally interact with the phosphates of ATP. In PKA, the phosphate groups interact with residues in the glycine-rich loop and the linker region. The interacting residues of the glycine-rich loop are invariant between PKA and PKC (52–55, GSFG), whereas those in linker region, from K72 (conserved) to E91(conserved), form a domain that varies among related protein kinases. Between PKA and PKC, there are notable differences in this region: in PKC, the sequence QDDD (81–84) replaces the KLKQ of PKA, with the possibility of unsatisfied H bonds in PKC and an alteration in the part of the protein that apposes the tip of ring D of balanol. We hypothesize that differences in the bonding interactions and in the structural complementarity of the kinases with the phosphate-mimicking regions of balanol contribute substantially to the selectivity of the 10" and 14" derivatives for PKA over PKC. Furthermore, we expect that a modest number of nonconservative amino acids substitu-

tions in the linker region (72–91) account for the distinctive interaction patterns of PKA and PKC with these derivatives.

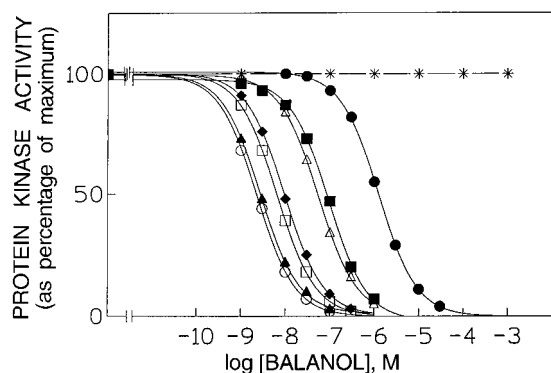
In summary, using balanol as a probe of the ATP binding region, we find similarities within the AGC group of protein kinases and significant diversity within the other subgroups of serine/threonine protein kinases. Within the AGC group, derivatives of balanol are able to distinguish PKA from PKC and to discriminate among PKC isoforms. Thus, the concept of a common catalytic core does not, alone, form an adequate basis for predicting which serine/threonine protein kinases will be inhibited by balanol. Our data indicate microscopic diversity among closely related proteins and support our view of balanol as a protean structure that may be modified to interact with considerable specificity and high affinity with the ATP-binding sites of a variety of serine/threonine protein kinases. Currently, we are testing additional balanol derivatives in an effort to refine our view of the structural features of balanol and protein kinases that contribute to the specificity of balanol and to the high affinity of balanol's interaction with the ATP-binding sites of serine/threonine protein kinases. In parallel with *in vitro* studies, we are also assessing the efficacy and specificity of balanol and congeners to inhibit protein kinases in intact cells (Gustafsson and Brunton, 1999).

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**Fig. 4.** Relative order of potency of balanol as an inhibitor of protein kinases. These theoretical curves were generated by using the calculated  $K_i$  values and computing the fractional occupancy (ordinate) by 10  $\mu$ M ATP in a hypothetical assay, using the equation: fractional occupancy =  $[L/K_d]/[1 + (L/K_d) + (I/K_i)]$ , where  $L = [ATP] = 10 \mu$ M,  $K_d$  is the dissociation constant for ATP;  $I$  is the concentration of balanol, and  $K_i$  is the inhibition constant for balanol. One hundred percent is the value in the presence of ATP alone (i.e.,  $L = 10 \mu$ M,  $I = 0$ ); other values are expressed as a percentage of this value.  $\circ$ , PKG;  $\blacktriangle$ , PKC $\beta$ II;  $\square$ , PKA;  $\blacklozenge$ , PKC $\alpha$ ;  $\triangle$ , p34<sup>cdc2</sup>;  $\blacksquare$ , CaMKII;  $\bullet$ , MAPK; and  $*$ , PhK, smMLCK, and CKII. The mean  $K_i$  and  $K_d$  values for balanol and ATP are summarized in Table 1.

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