

Differential and Selective Inhibition of Protein Kinase A and Protein Kinase C in Intact Cells by Balanol Congeners

ÅSA B. GUSTAFSSON and LAURENCE L. BRUNTON

Departments of Pharmacology and Medicine (Å.B.G., L.L.B.) and the Biomedical Sciences Graduate Program (Å.B.G.), University of California at San Diego, La Jolla, California

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ABSTRACT

The fungal metabolite balanol is a potent inhibitor of protein kinase A (PKA) and protein kinase C (PKC) in vitro that acts by competing with ATP for binding ($K_i \sim 4$ nM); congeners of balanol show specificity for PKA over PKC. We have characterized the effects of balanol and 10''-deoxybalanol in intact cells to determine whether these compounds cross the cell membrane and whether the potency and specificity noted in vitro are preserved in vivo. In neonatal rat myocytes and cultured A431 cells transiently transfected with a cyclic AMP response element-luciferase reporter construct, balanol inhibits the induction of luciferase activity by isoproterenol, indicating inhibition of PKA. Western analysis shows that both

balanol and 10''-deoxybalanol reduce phosphorylation of cAMP response element-binding protein in isoproterenol-stimulated A431 cells; inhibition is concentration dependent with an IC_{50} value of ~ 3 μ M. Balanol, but not 10''-deoxybalanol, inhibits phosphorylation of the myristoylated alanine-rich C kinase substrate protein, a PKC substrate, in phorbol ester-stimulated A431 cells ($IC_{50} \sim 7$ μ M). Our data demonstrate that balanol is a potent inhibitor of PKA and PKC in several whole-cell systems and causes no obvious toxicity. In addition, balanol congeners inhibit PKA and PKC with the specificity and potency predicted by in vitro experiments.

Balanol is a fungal metabolite produced by *Verticillium balanoides* (Kulanthaivel et al., 1993). The parent structure and a number of congeners (Fig. 1A) have been synthesized chemically (Lampe et al., 1994; Nicolaou et al., 1994). We have found that balanol is a potent inhibitor of cyclic AMP (cAMP)-dependent protein kinase (PKA) and protein kinase C (PKC) but not of two tyrosine protein kinases, pp60^{src} and the epidermal growth factor receptor kinase (Koide et al., 1995; Setyawan et al., 1999). In in vitro assays with purified components, balanol inhibits protein kinase activity by competing with ATP for binding at the enzyme's catalytic site (Koide et al., 1995). In fact, balanol interacts with PKA and PKC with an affinity ($K_i = 4$ nM) that is more than three orders of magnitude greater than that for ATP (Koide et al., 1995). We have recently taken advantage of the high affinity of the balanol-kinase interaction to isolate and analyze the structure of a crystal of the balanol-PKA complex, confirming that balanol binds in the ATP cleft of the catalytic core of PKA (Narayana et al., 1999).

Minor modification of the balanol structure produces congeners that exhibit substantial specificity toward PKA over

PKC. For instance, 10''-deoxybalanol (Fig. 1A) inhibits PKA with a K_i value of 4 nM and PKC with a K_i value of 640 nM in studies with purified enzymes in vitro (Koide et al., 1995; Setyawan et al., 1999). These data suggest that balanol might be a useful template on which a family of specific protein kinase inhibitors can be developed. However, biologically useful inhibitors need to enter cells and have desired effects at modest concentrations that do not injure the cells.

With these criteria in mind, we assessed the capacity of balanol to act in whole cells. Specifically, we have asked whether balanol inhibits PKA and PKC in whole cells, whether the inhibition occurs in the expected concentration range, and whether balanol and 10''-deoxybalanol exhibit, in intact cells, the specificity observed with purified enzymes in vitro. We also determined the effect of balanol on several receptor-G protein-linked systems that possess nucleotide triphosphate-binding sites and on cell viability. The results suggest that balanol and its derivatives are useful and specific inhibitors of protein kinases in vivo.

Experimental Procedures

Materials. Balanol and 10''-deoxybalanol were gifts from Dr. K. C. Nicolaou (Department of Chemistry and Biochemistry, University of

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ABBREVIATIONS: cAMP, cyclic AMP; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; MARCKS, myristoylated alanine-rich C kinase substrate; CRE, cyclic AMP response element; CREB, cyclic AMP response element-binding protein; TCA, trichloroacetic acid; ATF-1, activating transcription factor-1; DMEM, Dulbecco's modified Eagle's medium; PMA, phorbol-12-myristate-13-acetate; PAGE, polyacrylamide gel electrophoresis.

California at San Diego). cAMP response element (CRE)-luciferase was a gift from Dr. T. Hunter (Salk Institute, San Diego, CA). Lipofectamine was purchased from Life Technologies (Gaithersburg, MD). Immobilon P was obtained from Millipore Corporation (Bedford, MA). Protein A-Sepharose was obtained from Pharmacia (Piscataway, NJ).

Antibodies to phospho-cAMP response element-binding protein (CREB; Ser¹³³) and myristoylated alanine-rich C kinase substrate (MARCKS) were obtained from Upstate Biotechnology (Lake Placid, NY). Goat anti-rabbit horseradish peroxidase antibody was obtained from Life Technologies. [³²P]Orthophosphate was obtained from DuPont-New England Nuclear (Boston, MA). All other chemicals were reagent grade from Aldrich-Sigma (St. Louis, MO) or Calbiochem-Novabiochem (San Diego, CA).

Isolation of Neonatal Ventricular Myocytes; Cell Culture.

Myocytes were isolated from 1- to 2-day-old Sprague-Dawley rats using a collagenase-pancreatin digestion as described previously (Iwaki et al., 1990). Myocytes were purified by Percoll gradient centrifugation and plated onto dishes coated with 1% gelatin in Dulbecco's modified Eagle's medium (DMEM) and medium 199 (4:1) supplemented with 10% horse serum, 5% FBS, and penicillin/streptomycin (100 U/ml each). The myocytes were plated at a density of 5×10^5 cells/35-mm dish. After 24 h of culture, the cells were washed, placed in serum-free medium, and used for transient transfections. A431 human epidermal carcinoma cells were maintained in DMEM supplemented with 5% bovine calf serum.

Transient Transfection and Luciferase Assay. Neonatal myocytes were transfected using a modified calcium phosphate method as described previously (Ramirez et al., 1995) with 3 μ g of the reporter construct CRE-luciferase and 7 μ g of pBlueScript (to give a total of 10 μ g/35-mm dish). After transfections, the cells were incubated with balanol (3 μ M) or vehicle for 45 min in serum-free medium, and isoproterenol (10 μ M) or vehicle was added for 6 h. Myocytes were lysed, and luciferase activity was determined as described previously (Ramirez et al., 1995). A431 cells were plated onto 35-mm dishes and transfected with CRE-Luciferase by the Lipofectamine method according to the protocol supplied by the manufacturer (Life Technologies). Cells were incubated with balanol and then stimulated with isoproterenol as described above. Data are normalized to protein and expressed as a percentage of maximal luciferase activity.

Assessment of CREB Phosphorylation. A431 cells were serum starved for 2 h and then treated with balanol or congener for 45 min before the addition of 10 μ M isoproterenol or vehicle for 5 min. Cells were lysed in a buffer containing 50 mM β -glycerol phosphate, 1 mM EGTA, 10 mM MgCl₂, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, and 10 mM NaF. Proteins were separated on 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon P. Blots were blocked in 5% nonfat milk for 20 min at room temperature, incubated with phospho-CREB antibody overnight at 4°C and for 1 h at room temperature with goat anti-rabbit horseradish peroxidase secondary antibody, and then visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL). The addition of balanol 30 min before isoproterenol gave the same result as adding balanol 45 min beforehand.

Assessment of MARCKS Phosphorylation. A431 cells were labeled with ³²P_i (20 μ Ci/ml) in phosphate-free DMEM supplemented with 25 mM HEPES (pH 7.4 at 37°C) for 17 h at 37°C. Cells were incubated with balanol, staurosporine, or vehicle for 45 min, and then 100 nM phorbol-12-myristate-13-acetate (PMA) was added for 5 min. Cells were lysed by the addition of lysis buffer containing 50 mM β -glycerol phosphate, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 10 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml leupeptin. Lysates were cleared by centrifugation (10 min, 5000g), and an aliquot of the supernatant (1 mg protein) was incubated with 10 μ g of anti-MARCKS monoclonal antibody overnight at 4°C. The immunocomplex was incubated with protein A-Sepharose at 4°C for 2 h. The Sepharose beads were collected by centrifugation, washed three times with PBS, resuspended in SDS sample buffer, and subjected to SDS-PAGE (12%). Gels were dried and exposed to X-ray film at -70°C overnight.

Assessment of cAMP. A431 cells were pretreated with balanol for 45 min and then stimulated with 1 μ M isoproterenol for 10 min. Incubations were terminated by adding 5% ice-cold trichloroacetic

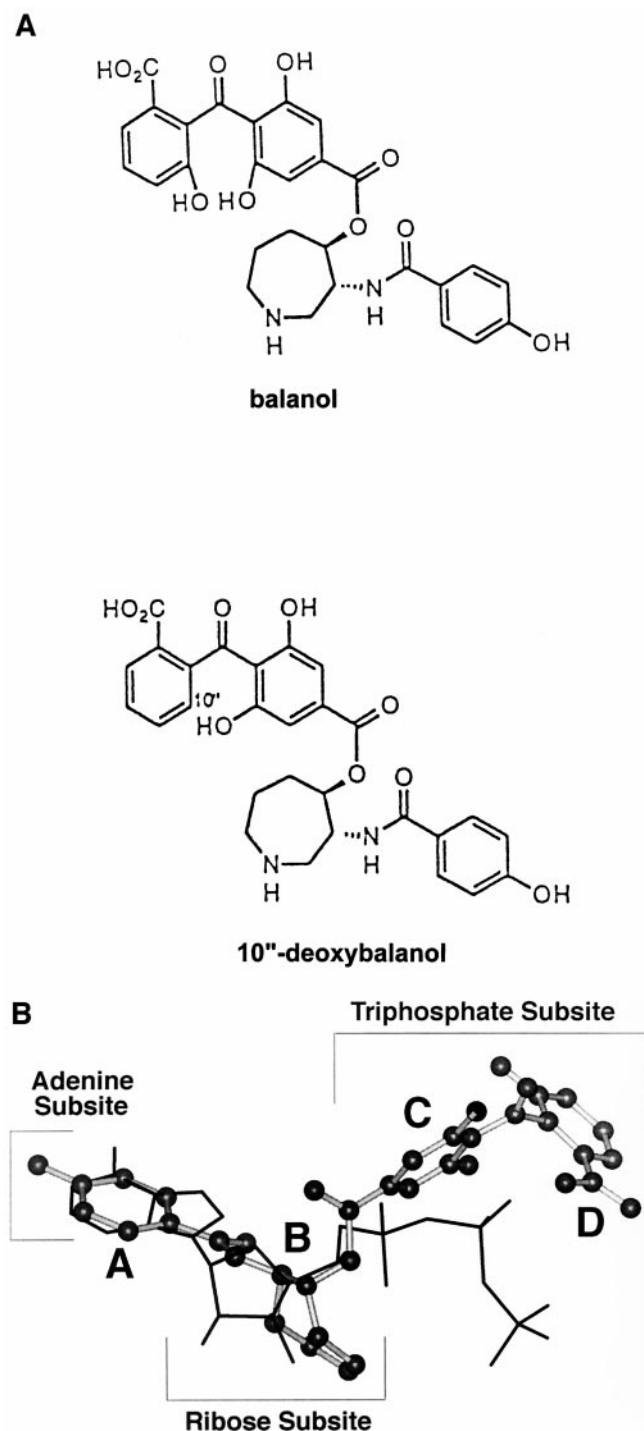


Fig. 1. A, structures of balanol and 10''-deoxybalanol. Balanol consists of three regions: benzophenone, hexahydroazepane, and 4-hydroxy benzoyl moieties. The benzophenone and hexahydroazepane moieties are connected through an ester linkage; the azepane and hydroxy-benzoyl moieties are interconnected by an amide linkage. B, comparison of the conformations of ATP and balanol when bound to the catalytic subunit of PKA. Ring A on balanol corresponds to the adenine of ATP, ring B to the region of the ribose, and rings C and D to the triphosphate of ATP (Narayana et al., 1999).

acid (TCA), and the TCA extracts were purified over Dowex AG50W \times 4 columns (200–400 mesh). cAMP content was determined according to the method of Gilman (1970). Data are corrected for recovery and expressed as picomole cAMP per milligram of cell protein.

Assessment of Phosphoinositide Hydrolysis. A431 cells were labeled overnight with *myo*-[^3H]inositol (5 $\mu\text{Ci}/\text{ml}$) in DMEM without serum. Cells were washed twice, and 10 mM LiCl was added 15 min before treatment with balanol. Balanol (10 μM) was added 45 min before 30 μM UTP. The stimulation (20 min) was terminated by aspiration of the medium and addition of ice-cold 5% TCA. The samples were extracted four times with water-saturated ether and then fractionated by anion exchange chromatography (Brown et al., 1985). Radioactivity in fractions corresponding to total inositol phosphates was quantified by liquid scintillation spectrometry. Data are expressed as counts per minute per plate.

Assessment of ATP. ATP content of perchloric acid extracts of A431 cells was estimated by an enzyme-linked fluorescence assay (Lowry and Passonneau, 1972).

Protein Determinations. Protein content was estimated by the method of Bradford (1976) using BSA as a standard.

Analysis of Data. Statistical analysis and graphing of data were performed with the program Prism 2.0 (GraphPAD Software, San Diego, CA). Differences were analyzed by one-way ANOVA followed by Student's *t* test. Values of *P* < .05 were considered to indicate significance.

Results

Preliminary Considerations. Balanol competes for the ATP site in the catalytic core of PKA and PKC. A comparison of the molecular structures of ATP and balanol when bound in ATP cleft of PKA reveals a striking similarity in the overall shape and size of the two molecules (Fig. 1B). Balanol consists of three fragments: the benzophenone, hexahydroazepane, and 4-hydroxy benzoyl moieties (Fig. 1A). The carboxylate, carbonyl, and hydroxyl groups of the benzophenone rings correspond to the three phosphates of ATP; the hexahydroazepane and the 4-hydroxy benzoyl amide moiety correspond to the ribose and adenine ring of ATP. Elimination of a hydroxyl group from the benzophenone ring produces 10⁻

deoxybalanol, a compound that shows two orders of magnitude selectivity for PKA over PKC (Fig. 1A).

In vitro, balanol inhibits PKA and PKC with K_i values of ≈ 4 nM, and ATP binds with K_d values of ≈ 20 μM . In cells, balanol would be competing with cellular ATP, ~ 2 mM (we find $[\text{ATP}] = 20.4 \pm 1.7$ nmol/mg protein in A431 cells [mean \pm range, *n* = 2], similar to the value reported for the ATP content of rat myocytes; that value corresponds to 2–3 mM). Using the expression,

Fractional occupancy of PKA by balanol =

$$\frac{I}{I + K_i + L \cdot \frac{K_i}{K_d}} \quad (1)$$

where I = [balanol], K_i = 4 nM, L = [ATP] = 2 mM, and K_d = 20 μM , we calculated that balanol would occupy 50% of ATP-binding sites on PKA and PKC at intracellular concentrations in the low micromolar range.

We first tested balanol for its effects on cell viability and transmembrane signaling. As judged by trypan blue exclusion and the maintenance of cell shape, balanol (10 μM for 7 h) did not produce noticeable toxicity; indeed, concentrations as high as 30 μM (highest concentration tested) had no obvious deleterious effects.

Because balanol is a nucleotide triphosphate congener, we considered the possibility that balanol would interfere with transmembrane signal transduction (at GTP sites on G proteins), at the ATP-binding site of adenylyl cyclase, at the phosphatidylinositol bisphosphate-binding site of phospholipase C, and at the ATP/UTP-binding site of the P_{2Y2} purinergic receptor. Balanol (10 μM) did not reduce cAMP accumulation (in pmol/mg of total cell protein) in A431 cells stimulated with the β -agonist isoproterenol (1 μM): control, 25.3 ± 0.2 ; isoproterenol, 1625 ± 19 ; and isoproterenol plus balanol, 1703 ± 27 . Similarly, balanol (10 μM) did not stimulate phosphatidylinositol hydrolysis or inhibit the effect of UTP (30 μM) to stimulate phosphatidylinositol hydrolysis

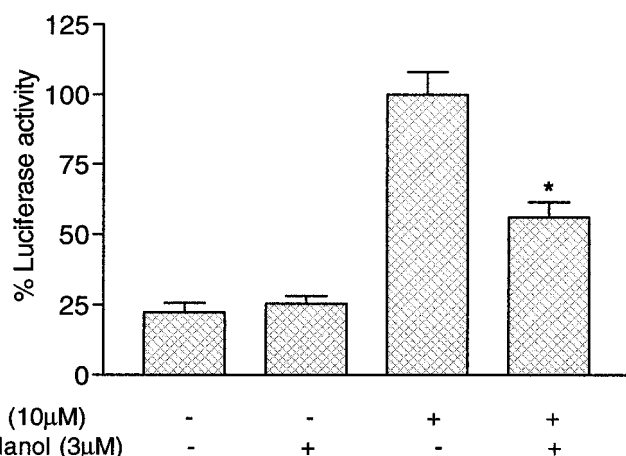


Fig. 2. Balanol inhibits expression of CRE-luciferase in neonatal myocytes. Neonatal myocytes, transiently transfected with a CRE-luciferase reporter gene, were incubated with diluent or balanol (3 μM) for 45 min before stimulation with 10 μM isoproterenol (Iso) for 6 h. Balanol produced a significant decrease in the induction of luciferase in isoproterenol-stimulated cells (**p* < .001). Luciferase activity was calculated as the mean of triplicate samples from duplicate experiments.

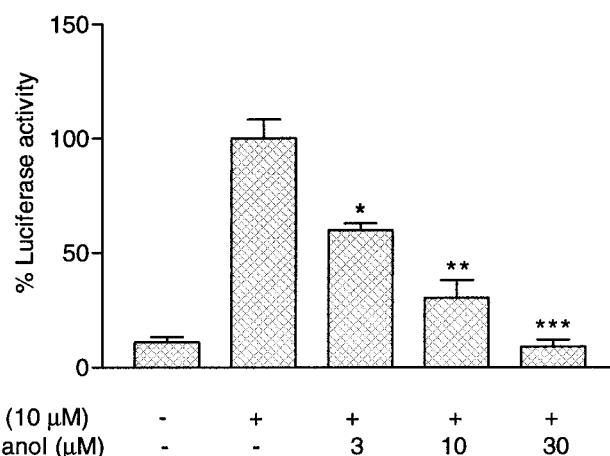


Fig. 3. Balanol inhibits expression of CRE-luciferase in cultured A431 cells. A431 cells, transiently transfected with CRE-luciferase reporter gene, were incubated with increasing concentrations of balanol for 45 min before the addition of isoproterenol (10 μM) for 6 h. Data are mean \pm S.E.M. of duplicate samples from three independent experiments. **p* < .001; ***p* < .0001; ****p* < .0001, compared with isoproterenol-stimulated cells.

(cpm/plate) in A431 cells: UTP, 2.4 ± 0.7 -fold increase over control; UTP plus balanol, 2.3 ± 0.6 -fold increase over control. We interpret these data to indicate that balanol is without noticeable effect on the P_{2Y2} receptor or on the G proteins and effectors responsible for hormone-stimulated production of inositol phosphates and cAMP, pathways that lead to the activation of PKC and PKA *in vivo*. Subsequent experiments were designed to test the effects of balanol on PKA and PKC activities in whole cells.

Inhibition of CRE-Luciferase Activation. To determine whether balanol is cell permeable and capable of inhibiting PKA within the appropriate concentration range, we studied the ability of balanol to inhibit induction of luciferase in cells transiently transfected with the construct CRE-luciferase, a sensitive reporter of elevated cAMP. In neonatal rat myocytes, isoproterenol caused a more than 4-fold increase in inducible luciferase activity (Fig. 2). Balanol by itself had no effect on luciferase activity, but in myocytes pretreated with $3 \mu\text{M}$ balanol, the induction of luciferase activity was decreased to about 55%, indicating inhibition of PKA.

This effect of balanol was not confined to neonatal rat myocytes. In cultured A431 cells transiently transfected with the CRE-luciferase construct, balanol also reduced isoproterenol-stimulated luciferase induction. The effect of balanol was concentration dependent, with an IC_{50} value of $\sim 3 \mu\text{M}$ (Fig. 3). Treatment of cells with $30 \mu\text{M}$ balanol completely inhibited luciferase induction by isoproterenol. Thus, the effect of balanol is not limited to a single cell type and shows concentration dependence in the range predicted by *in vitro* experiments.

Inhibition of CREB Phosphorylation. Luciferase induction as an endpoint is distant in time and place from balanol's putative target, PKA. As a more proximal assessment of PKA activity, we measured the phosphorylation state of a PKA substrate, the transcriptional activator CREB. PKA phosphorylates CREB at Ser^{133} (Gonzalez and Montminy, 1989). We assessed phosphorylation of CREB by Western analysis using an antibody specific for the Ser^{133} -phos-

phorylated form of CREB (see *Experimental Procedures*). Treatment of A431 cells with isoproterenol increased CREB phosphorylation at Ser^{133} (Fig. 4A). The addition of balanol before isoproterenol caused a concentration-dependent decrease in CREB phosphorylation (IC_{50} value is somewhat less than $3 \mu\text{M}$). The antibody used to detect phospho-CREB also detects phosphorylation of activating transcription factor-1 (ATF-1; the band below CREB), a related transcription factor that shares sequence identity surrounding Ser^{133} . ATF-1 cross-reacts with the anti-CREB used and is also phosphorylated by PKA (Liu et al., 1993; Shimomura et al., 1996). Balanol decreased phosphorylation of ATF-1 along roughly the same concentration-dependence curve.

The balanol derivative $10''$ -deoxybalanol (Fig. 1B) also is a PKA inhibitor *in vitro*, equipotent with balanol ($K_i = 4.7 \text{ nM}$ for balanol and $= 3.9 \text{ nM}$ for $10''$ -deoxybalanol; Koide et al., 1995). $10''$ -Deoxybalanol likewise reduced phosphorylation of CREB in a concentration-dependent manner (Fig. 4B; IC_{50} value is somewhat less than $3 \mu\text{M}$). Thus, $10''$ -deoxybalanol and balanol are both potent inhibitors of PKA in the intact cell, exhibiting potencies consistent with their inhibition constants determined *in vitro*.

Inhibition of MARCKS Protein Phosphorylation. To determine whether balanol could inhibit PKC in the intact cell, we looked at the capacity of balanol to inhibit phosphorylation of the MARCKS protein, which is phosphorylated after activation of cellular PKC (Rosen et al., 1989). In A431 cells labeled with *ortho*- ^{32}P , treatment of cells with PMA increased phosphorylation of the MARCKS protein by about 2.5-fold (Fig. 5A). Balanol ($10 \mu\text{M}$) reduced this effect of PMA by 60%, indicating an IC_{50} value in the low micromolar range (estimated to be $7 \mu\text{M}$; data not shown). On the other hand, the addition of $10''$ -deoxybalanol had virtually no effect on PMA-stimulated MARCKS phosphorylation (Fig. 5B), in keeping with the specificity observed *in vitro*. [Using the *in vitro* data of Koide et al. (1995) for PKC ($K_i = 4 \text{ nM}$ for balanol and 640 nM for $10''$ -deoxybalanol) and assuming that the balanol derivatives equilibrate into cells to a final con-

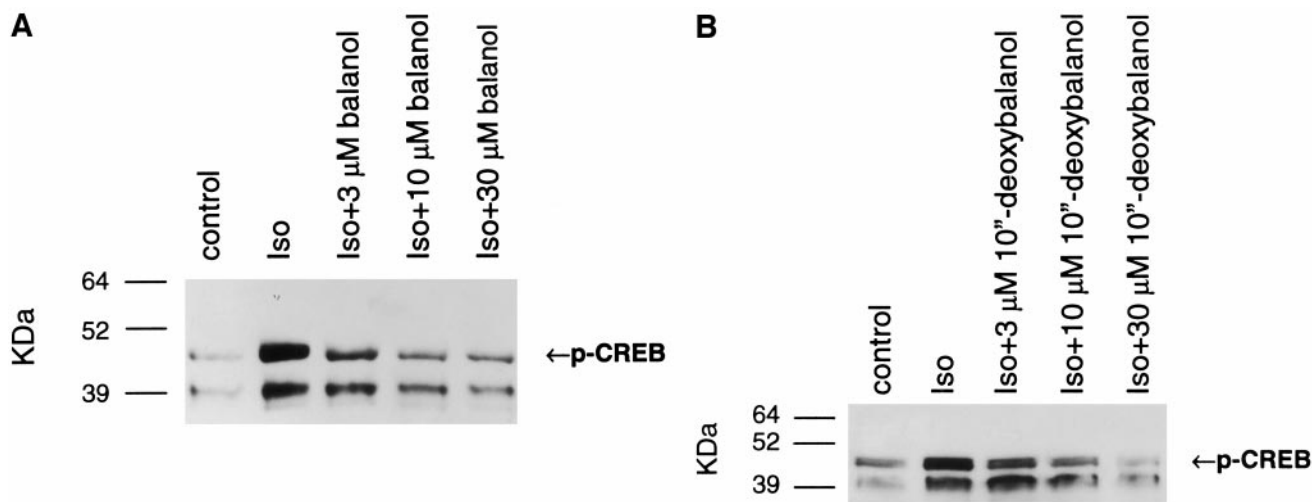


Fig. 4. A, balanol inhibits CREB phosphorylation. A431 cells were pretreated with increasing concentrations of balanol as described in the legend to Fig. 3 and then stimulated with isoproterenol ($10 \mu\text{M}$) for 6 h. Cell lysates were subjected to SDS-PAGE, followed by immunoblot (Western) analysis using an antibody against phosphorylated CREB. Isoproterenol treatment resulted in increased CREB phosphorylation; balanol inhibited phosphorylation in a concentration dependent manner. Blot is representative of three replicate experiments. B, $10''$ -deoxybalanol inhibits CREB phosphorylation. A431 cells were incubated with increasing concentrations of $10''$ -deoxybalanol and then with isoproterenol, as described above. $10''$ -Deoxybalanol inhibited CREB phosphorylation in a concentration-dependent manner. Western blot is representative of three replicate experiments.

centration of 10 μM , we estimate the fractional occupancy of PKC by balanol as 96% and by 10''-deoxybalanol as 13%.]

Discussion

We set out to determine whether balanol congeners are effective inhibitors of PKA and PKC in intact cells and whether balanol and its derivatives show the specificity predicted by experiments on purified enzymes. There are a number of factors that might have contributed to variation from the calculated effective concentration range: failure of balanol to enter cells, partitioning of the drug into the membrane or other cellular compartments that would effectively lower its concentration in the vicinity of cellular PKA and PKC, metabolism of the drug, and excess PKA and PKC, similar to "spare" receptors, such that activation of only a small fraction of the protein kinase suffices to produce a maximal effect. Our data show that such issues do not limit balanol's efficacy in two cell systems. We find that balanol, in low micromolar

concentrations, inhibits cellular PKA and PKC, as predicted. Furthermore, a congener that displays specificity for PKA over PKC in vitro shows the same selectivity in vivo.

The effects of balanol that we assessed involved balanol concentrations as high as 30 μM and exposure times as long as 7 h. Visual microscopic analysis and trypan blue staining indicated no obviously toxic effects of balanol on either rat neonatal cardiac myocytes or cultured human A431 cells. In both of the cell types, we were able to demonstrate that balanol does not interfere with the signal transduction pathways (β receptor- G_s -adenylyl cyclase-cAMP, P_{2Y2} receptor- G_q -phospholipase C-inositol trisphosphate) by which hormones stimulate second messenger production and, hence, activate PKA and PKC.

The fact that a modification as slight as removal of the 10'-hydroxyl changes the selectivity of balanol suggests that balanol may be modified to produce very selective protein kinase inhibitors that can take advantage of microheterogeneities in the catalytic cores of closely related protein kinases (Setyawan et al., 1999). It is generally a mistake to overestimate the specificity and selectivity of ATP-analog protein kinase inhibitors. However, 10''-deoxybalanol shows considerably more selectivity for PKA than the popular PKC inhibitor staurosporin exhibits toward PKC [in our hands, staurosporine inhibits PKC with a K_i value of 19 nM and inhibits PKA almost equally well, $K_i = 35$ nM, a negligible level of selectivity (Koide et al., 1995)].

In summary, we have demonstrated that balanol and 10''-deoxybalanol are effective, nontoxic inhibitors of PKA and PKC in whole cells, in the concentration range and with the selectivity predicted by studies on purified enzymes in vitro. We are continuing to evaluate other derivatives of balanol in vitro and in vivo with the idea that balanol may be a protean structure, derivatives of which may provide effective and relatively specific inhibitors of a number of serine/threonine protein kinases in biological systems.

Acknowledgments

We appreciate the gift of balanol and 10''-deoxybalanol from Dr. K.C. Nicolaou and information on the structures of PKA-bound ATP and balanol from Dr. Susan S. Taylor (both of the Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, CA).

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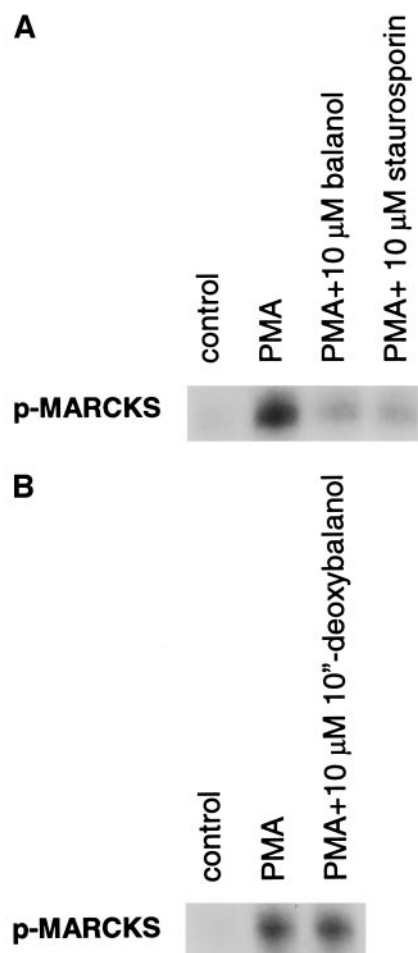


Fig. 5. A, balanol inhibits MARCKS phosphorylation. A431 cells, labeled with *ortho*- ^{32}P , were pretreated with 10 μM balanol or 10 μM staurosporine (positive control) for 45 min before exposure to 100 nM PMA for 5 min. The MARCKS protein was immunoprecipitated using a human monoclonal antibody and analyzed by SDS-PAGE followed by autoradiography. Autoradiogram is representative of three replicate experiments. B, 10''-deoxybalanol does not inhibit MARCKS phosphorylation. A431 cells were pretreated with 10 μM 10''-deoxybalanol for 45 min before stimulation with 100 nM PMA for 5 min. MARCKS phosphorylation was determined as described above. Autoradiogram is representative of two replicate experiments.

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Send reprint requests to: Dr. Åsa B. Gustafsson, Department of Pharmacology 0636, University of California San Diego, School of Medicine, La Jolla, CA 92093-0636. E-mail, agustafsson@ucsd.edu
