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

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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 ~ 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 μM. 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} ~ 7 μM). 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 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.

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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.
region of the ribose, and rings C and D to the triphosphate of ATP. Ring A on balanol corresponds to the adenine of ATP, ring B to the formations of ATP and balanol when bound to the catalytic subunit of PKA. Ring 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 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 μg of the reporter construct CRE-luciferase and 7 μg of pBlueScript (to give a total of 10 μg/35-mm dish). After transfections, the cells were incubated with balanol (3 μM) or vehicle for 45 min in serum-free medium, and isoproterenol (10 μM) 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 μM isoproterenol or vehicle for 5 min. Cells were lysed in a buffer containing 50 mM β-glycerophosphate, 1 mM EGTA, 10 mM MgCl₂, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/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, (20 μCi/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-glycerolphosphate, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 10 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. Lysates were cleared by centrifugation (10 min, 5000g), and an aliquot of the supernatant (1 mg protein) was incubated with 10 μg 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 μM isoproterenol for 10 min. Incubations were terminated by adding 5% ice-cold trichloroacetic acid. Cells were lysed in a buffer containing 50 mM KCl, 50 mM glucose, 10 mM HEPES (pH 7), 10 μg/ml leupeptin, and 10 mM NaF. To determine the intracellular cAMP levels, cells were incubated with balanol 30 min before isoproterenol as described above. cAMP was quantified using a cAMP enzyme immunoassay kit (Amersham, Arlington Heights, IL).
acid (TCA), and the TCA extracts were purified over Dowex AG50W × 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 μCi/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 Passoneau, 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 moiety (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 [ATP] = 20.4 ± 1.7 nmol/mg protein in A431 cells [mean ± 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,

\[
I = \frac{I + K_i + L \cdot K_d}{K_d}
\]

where $I = [\text{balanol}]$, $K_i = 4$ nM, $L = [\text{ATP}] = 2$ 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 adenyl cyclase, at the phosphatidylinositol bisphosphate-binding site of phospholipase C, and at the ATP/UTP-binding site of the P2_Y2 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.

![Inhibition of PKA and PKC by Balanol Congeners In Vivo](image)

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.

![Inhibition of PKA and PKC by Balanol Congeners In Vivo](image)

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 ± 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 P2Y2 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 subsequent experiments. We 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 constitutive 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 μM 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 IC50 value of ~3 μM (Fig. 3). Treatment of cells with 30 μM 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 Ser133 (Gonzalez and Montminy, 1989). We assessed phosphorylation of CREB by Western analysis using an antibody specific for the Ser133-phosphorylated form of CREB (see Experimental Procedures). Treatment of A431 cells with isoproterenol increased CREB phosphorylation at Ser133 (Fig. 4A). The addition of balanol before isoproterenol caused a concentration-dependent decrease in CREB phosphorylation (IC50 value is somewhat less than 3 μM). 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 Ser133. 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 (Kd = 4.7 nM for balanol and = 3.9 nM for 10'-deoxybalanol; Koide et al., 1995). 10'-Deoxybalanol likewise reduced phosphorylation of CREB in a concentration-dependent manner (Fig. 4B; IC50 value is somewhat less than 3 μM). 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-32P, treatment of cells with PMA increased phosphorylation of the MARCKS protein by about 2.5-fold (Fig. 5A). Balanol (10 μM) reduced this effect of PMA by 60%, indicating an IC50 value in the low micromolar range (estimated to be 7 μM; 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 (Kd = 4 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 μM) 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 PKC 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 βγ-adenyl cyclase-CAMP, P2Y2 receptor-Gi- 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 Kᵢ value of 19 nM and inhibits PKA almost equally well, Kᵢ = 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.

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References


Fig. 5. A, balanol inhibits MARCKS phosphorylation. A431 cells, labeled with ortho-23P, 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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