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PRINCIPAL INVESTIGATOR: Theodore Slotkin, Ph.D.

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, North Carolina 27710

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6. AUTHOR(S)
Theodore Slotkin, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University Medical Center
Durham, North Carolina 27710

E-MAIL:
t.slotkin@duke.edu

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13. ABSTRACT *(Maximum 200 Words)*

This study explores β-adrenoceptors on breast cancer cells as a therapeutic target. MDA-MB-231 human breast cancer cells express high β_2-adrenoceptor levels that are linked to inhibition of mitosis through the production of cyclic AMP. Receptor stimulation by β-agonists, or administration of the phosphodiesterase inhibitor, theophylline, led to mitotic arrest and a consequent reduction in cell number, accompanied by altered cellular morphology. We showed that the effects were mediated by the cellular intermediate, cyclic AMP; the responses did not desensitize even with prolonged drug exposure and could be enhanced by the coadministration of glucocorticoids, such as dexamethasone, that enhance β-adrenoceptor-mediated cyclic AMP production. The largest effects were seen for theophylline, which exerted additional actions mediated through adenosine receptors and the production of reactive oxygen species. Theophylline evoked specific changes in the expression/function of membrane-bound adenylyl cyclase activity, effects that are likely to contribute to sustained reactivity of these cells to other cyclic AMP-related inhibitors of cell proliferation, such as β-adrenergic agonists. Strategies focusing on cell surface receptors, such as β-adrenoceptors, may combat cancers that are unresponsive to hormonal agents, or that have developed multidrug resistance, since the drugs do not need to penetrate the cell membrane to work.

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INTRODUCTION

A number of human breast cancers manufacture β-adrenergic receptors, for which there are drugs available that stimulate or inhibit their activity. The current study investigated the regulation and function of these receptors and identified factors that can promote the ability of the receptors to influence cell division. In normal cells, the receptors are linked to the genes that control cell division only during a discrete developmental period in which they first enhance, and then terminate cell replication. We hypothesized that the redifferentiation that accompanies carcinogenesis would render cells responsive in the same way as are developing cells, but without the subsequent loss of reactivity that normally occurs as cells mature. There were three Specific Aims: (1) to determine whether β-receptors are linked to protooncogene expression and to evaluate the ability of the receptors to downregulate or desensitize upon exposure to a stimulant (isoproterenol); (2) to determine whether factors that promote the activity of β-receptor signaling lead to altered protooncogene expression and inhibition of cell replication — inhibitors cAMP breakdown (theophylline), or glucocorticoids (dexamethasone); (3) to determine whether stimulation or blockade of β-receptors can be used to control cell replication. In extending these results to detection and novel treatment paradigms, biopsy can readily identify the presence of β-receptors on breast cancer cells, indicating patients in whom xanthine derivatives and β-receptor agonists may serve as a useful addition to surgical, chemotherapeutic and radiation interventions. Perhaps most importantly, because β-adrenergic control of protooncogene expression is "upstream" from cell cycle control, interventions aimed at receptor-driven events should still operate even after multidrug resistance appears.

BODY

Relationship of progress to each task described in the original Statement of Work:

The original Statement of Work contained the following tasks:

Task 1 — Dose-response and time-response curves for isoproterenol sensitization or desensitization of β-receptors and adenylyl cyclase. Studies conducted in year 1.

Task 2 — Dose-response and time-response curves for isoproterenol induction of c-*fos* protooncogene expression. Studies conducted in year 3.

Task 3 — Dose-response and time-response curves for isoproterenol effects on DNA synthesis and cell number. Studies conducted in year 1.

Task 4 — Dose-response and time-response curves for isoproterenol effects on nuclear labeling. Studies conducted in years 1 and 3.

Task 5 — Dose-response and time-response curves for isoproterenol sensitization or desensitization of β-receptors and adenylyl cyclase in the presence or absence of theophylline; and in the presence or absence of dexamethasone — contains all the elements of Tasks 1, 2, 3. Studies conducted in years 1 and 2.

Task 6 — Ability of propranolol to block stimulatory effects of isoproterenol — contains all the elements of Tasks 1, 2, 3. Studies conducted in year 1.

In the first year, we chose to concentrate first on the demonstration that isoproterenol treatment could indeed interfere with breast cancer cell replication. We switched from the originally-proposed cell line (CG-5) to another line (MDA-MB-231) when it became apparent that the latter actually had a much higher concentration of β-adrenoceptors, was more readily available, and had a more demonstrable, widespread distribution in human cancers. To remain within a unitary set of techniques for each phase of the study, our first year concentrated on cell replication, cell number, adenylyl cyclase and receptor regulation as the targets (Task 1, Task 3, part of Task 4, and the corresponding sections of Tasks 5 and 6). In the course of the first year's studies, we made the unusual observation that, among all the treatments evaluated, theophylline had by far the greatest inhibitory effect on mitosis. Accordingly, we shifted our priorities to concentrate on elaborating the mechanism of action of theophylline as the primary task for year 2 (it was originally scheduled for year 3), and shifting the remaining two tasks to the third year. In conducting the protooncogene studies during year 3, however (Task 2 and the remaining part of Task 4), we ran into major difficulties. The cell line did not show significant levels of c-fos mRNA as detected with the standard human probe, nor did PCR amplification result in measurable induction over the small baseline values. It is possible, or even likely, that the mutations associated with the MDA-MB-231 cell line involve changes in the sequence for the c-fos gene that make the standard cDNA probe useless. However, at the same time, our results with the theophylline studies in year 2 were spectacular -- complete mitotic arrest and a decrease in cancer cell viability. We therefore refocussed the remainder of year 3 on identifying what properties of the theophylline molecule rendered it so successful as an anticancer agent; our studies on reactive oxygen species were not part of the original set of tasks, but nevertheless represented an appropriate (and exciting) scientific direction to conclude the project, in lieu of the unsuccessful attempt to characterize protooncogene responses.

The body of this report is divided into three sections, corresponding to each of the three years of the project. Each section contains an Introduction to the tasks addressed in that year, with the appropriate Methods and Results. Discussion of the results obtained, their importance and implications, appear in Conclusions.

Year 1

In addition to their role as neurotransmitters and "stress" hormones, catecholamines play a trophic role in the control of cell replication and differentiation in target cells that express adrenergic receptors. Lower organisms, such as sea urchins, overexpress norepinephrine, epinephrine and other biogenic amines during critical developmental periods in which these amines control cell replication and differentiation [6]. In mammals, "spikes" of adrenergic activity also modulate the rate of cell replication and differentiation and thus control the architectural modeling of adrenergic target tissues [11,28,44,46,57]. The importance of adrenergic control of mammalian cell development has recently been pointed out by the lethal effects of gene knockouts that eliminate the ability to synthesize norepinephrine or to express β-receptors; these animals die *in utero* from disruption of cardiac cell replication/differentiation and consequent dysmorphogenesis [38,53,68]. The critical period for adrenergic control of these events

terminates as cells exit mitosis and approach terminal differentiation, so that the sensitivity to adrenergic stimulation of cell replication disappears in adulthood except for a few tissues that undergo continual renewal [11,46,60,67].

It is thus of critical importance that, with carcinogenic redifferentiation, many cell types, including epithelial cancers and cancers of secretory cells, re-express β-adrenergic receptors [7,27,29,33,37], which can once again resume their role in the control of cell replication [33,36,37]. In some cell lines, β-adrenergic stimulation elicits a small, promotional effect on cell replication [36,37,62], whereas in others, stimulation of these receptors and the consequent rise in intracellular cAMP levels inhibit mitosis [9,33]. β-Adrenoceptors on cancer cells thus recapitulate both the promotional and inhibitory roles of these receptors in cell replication seen in the development of normal cells [11,16,44,46,47]. Accordingly, it might be feasible to use β-adrenoceptor agonists or antagonists as pharmacologic interventions to control the replication of cancer cells. Indeed, short-term isoproterenol treatment of PC-3 prostate cancer cells inhibits DNA synthesis through β-receptor-mediated increases in cAMP [33], and direct administration of membrane permeable cAMP analogs inhibits tumorigenesis of MCF-7 breast cancer cells [9].

Receptor downregulation and desensitization are major problems limiting the potential use of β-receptor agonists to control cell replication. Ordinarily, prolonged receptor stimulation uncouples receptors from response elements (desensitization) and leads to internalization and sequestration of receptor proteins (downregulation), limiting the intensity and duration of cell stimulation [50]. During normal development, however, we have found that these processes are poorly developed so that responses are maintained or enhanced with agonist treatment [19,65,66]. This raises the possibility that loss of response may not occur in cancer cells as well. In the current study, we evaluate that hypothesis using MDA-MB-231 cells, a human breast cancer line that expresses high levels of β-adrenoceptors [55]. We report that prolonged β-agonist administration maintains inhibition of DNA synthesis and suppresses cell replication even when only a small proportion of the receptors remain, so that desensitization and downregulation do not limit the effect. We also show that effects are augmented by glucocorticoids, just as is true for normal cells during development [43], and also by inhibition of cAMP breakdown by theophylline.

Methods

MDA-MB-231 cells (Duke University Comprehensive Cancer Center, Durham, NC) were seeded at a density of 10^6 cells per 100 mm diameter dish and maintained in modified Minimum Essential Medium containing Earle's salts, 5% fetal bovine serum, 2 mM glutamine, 100 IU/ml of penicillin, 0.1 mg/ml of streptomycin and 5 μg/ml of insulin (all from Gibco, Grand Island, NY). Cells were incubated with 7.5% CO_2 at 37° C and the medium was changed every 24h. Cells were examined at 100× magnification for counting and morphological features. Each experiment was repeated several times with separate batches of cells, after an average of five passages. Each passage and preparation was verified for morphology, growth rate and the expression and/or function of β-adrenoceptors. Treatment effects were always compared against concurrent control cultures from the same passage and batch of cells. Except as otherwise indicated, all drugs were

obtained from Sigma Chemical Co. (St. Louis, MO).

DNA synthesis and content. To initiate the measurement of DNA synthesis, the medium was changed to include 1 μCi/ml of [^{3}H]thymidine (specific activity, 2 Ci/mmol; New England Nuclear, Boston, MA). Incubations were carried out for 1h in the presence or absence of the appropriate drugs. At the end of that period, the medium was aspirated and cells were harvested in 3.5 ml of ice-cold water. Duplicate aliquots of each sample were treated with 10% trichloroacetic acid and sedimented at 1000 _ *g* for 15 min to precipitate macromolecules and the resultant pellet was washed once with additional trichloroacetic acid and with 75% ethanol. The final pellet was then hydrolyzed with 1 M KOH overnight at 37° C, neutralized with HCl and the DNA was then precipitated with ice-cold 5% trichloroacetic acid and sedimented at 1000 _ *g* for 15 min. The pellet from this final step was hydrolyzed in 5% trichloroacetic acid for 15 min at 90° C, resedimented, and an aliquot of the supernatant solution counted for [^{3}H]thymidine incorporation. Another aliquot was assayed for DNA spectrophotometrically by absorbance at 260 nm. Previous work has demonstrated quantitative recovery of DNA by these techniques [3]. Incorporation values were corrected to the amount of DNA present in each culture to provide an index of DNA synthesis per cell.

β-Adrenoceptor binding. The medium was removed and cells were washed once with ice-cold, calcium- and magnesium-free Earle's balanced salt solution. Fresh solution was added and the cells were scraped off the dish and sedimented at 40,000 × *g* for 15 min. The pellet was resuspended (Polytron, Brinkmann Instruments, Westbury, NY) in 10 mM $MgCl_2$, and 50 mM Tris (pH 7.4) and the homogenate was sedimented at 40,000 × *g* for 15 min. The pellets were dispersed with a homogenizer (smooth glass fitted with a Teflon pestle) in the same buffer.

Each assay contained membrane suspension corresponding to ≈5 μg of protein and 67 pM [^{125}I]iodopindolol (specific activity 2200 Ci/mmol, New England Nuclear) in a final volume of 250 μl of 145 mM NaCl, 2 mM $MgCl_2$, 20 mM Tris (pH 7.5) and 1 mM ascorbate. Nonspecific binding was evaluated with identical samples containing 100 μM isoproterenol, and was typically 15% of the total binding. In some experiments, displacement of ligand binding was carried out with the specific β_1-receptor antagonist, CGP20712A (Research Biochemicals International, Natick, MA) to identify the receptor subtype present on MDA-MB-231 cells. Scatchard determinations to identify changes in receptor number (B_{max}) or affinity (K_d) were carried out over a range of [^{125}I]iodopindolol concentrations from 0.02 to 1 nM.

Adenylyl cyclase activity. Cell membranes were prepared by the same procedure as for β-receptor binding, except that the buffer consisted of 250 mM sucrose, 1 mM EGTA, 10 mM Tris (pH 7.4). Aliquots of membrane preparation containing ≈20 μg protein were then incubated for 30 min at 30° C with final concentrations of 100 mM Tris-HCl (pH 7.4), 10 mM theophylline, 1 mM adenosine 5'-triphosphate, 10 mM $MgCl_2$, 1 mg bovine serum albumin, and a creatine phosphokinase-ATP-regenerating system consisting of 10 mM sodium phosphocreatine and 8 IU phosphocreatine kinase, and 10 μM GTP in a total volume of 250 μl. The enzymatic reaction was stopped by placing the samples in a 90-100° C water bath for 5 min, followed by sedimentation at 3000 × *g* for 15 min, and the supernatant solution was assayed for cAMP using

radioimmunoassay kits (Amersham Corp., Chicago, IL). Preliminary experiments showed that the enzymatic reaction was linear well beyond the assay time period and was linear with membrane protein concentration; concentrations of cofactors were optimal and, in particular, the addition of higher concentrations of GTP produced no further augmentation of activity. In addition to evaluating basal activity, the maximal total activity of the adenylyl cyclase catalytic unit was evaluated with the response to 10 mM $MnCl_2$ [8].

The contributions of G-protein-linked processes to adenylyl cyclase were evaluated in two ways. First, to determine the net G-protein-linked response of adenylyl cyclase activity with maximal activation of all G-proteins, samples were prepared containing 10 mM NaF in the presence of GTP [8]. Second, β-adrenoceptor-targeted effects mediated through the G-proteins were evaluated with 100 μM isoproterenol in the presence of GTP. The concentrations of all the agents used here have been found previously to be optimal for effects on adenylyl cyclase and were confirmed in preliminary experiments [8,35].

Data analysis. Data are presented as means and standard errors. For each study, treatment-related differences were first evaluated by a global ANOVA, incorporating all variables in a single test. For studies of adenylyl cyclase activity, multiple measurements were made from the same membrane preparation since several different stimulants compared; in that case, stimulant was considered a repeated measure. For studies of blockade of one drug by another, or of additive or synergistic effects, the combined effects were evaluated by two-factor ANOVA with the working hypothesis dependent upon a significant interaction between the two treatments. Where significant treatment effects were identified with the global test, individual differences between treatment groups were established with Fisher's Protected Least Significant Difference.

Scatchard plots were fitted by linear regression analysis and treatment-related differences were first compared by ANCOVA. Differences in maximal binding capacity (B_{max}) and the equilibrium dissociation constant (K_d, the reciprocal of receptor affinity) were then evaluated using Fisher's Protected Least Significant Difference.

Significance for main treatment effects was assumed at $p < 0.05$ and interaction terms were considered significant at $p < 0.1$ [49]. For convenience, some data are presented as a percentage of control values but statistical significance was always assessed on the unmanipulated data. Where multiple time points are presented in the same graph, the control groups are given as a single value (100%), but statistical comparisons were conducted only with the time-matched group appropriate to each treatment.

Results

MDA-MB-231 cells were in log-phase growth from 1 to 4 days after plating (Fig. 1). Over this span, DNA synthesis was maintained at a nearly constant rate and the number of cells, indicated by total DNA content, rose substantially. The increase in confluence between 1 and 4 days (more than double) was larger than the increase in DNA content (65%), indicating that cell enlargement was also occurring over this span. Drug treatments were initiated after one day in culture and were terminated at various times during log-phase growth, no later than four days in culture.

Addition of as little as 1 nM of isoproterenol to the medium produced immediate and robust inhibition of DNA synthesis (Fig. 2). The effect was maximal at 100 nM isoproterenol and was maintained throughout a 48h exposure. At the end of that period, isoproterenol-treated cells showed a significant reduction in the number of cells, assessed by DNA content. To demonstrate that the effects of isoproterenol were mediated through β-adrenoceptors stimulating the production of cAMP, a comparison was made with the membrane permeable cAMP analog, 8-Br-cAMP, and with the effects of the β-receptor antagonist, propranolol (Fig. 3). Isoproterenol and 8-Br-cAMP were equally effective toward DNA synthesis, and the effect of isoproterenol was completely blocked by propranolol. Propranolol by itself had no effect.

In developing tissues, glucocorticoid administration can sensitize cells to β-adrenoceptor agonists by inducing receptor formation and by enhancing signaling components of the adenylyl cyclase cascade (Slotkin et al., 1994a). Accordingly, we examined whether dexamethasone enhances the ability of isoproterenol to inhibit DNA synthesis and to reduce the number of cells (Fig. 4). By itself, a 24h or 48h pretreatment with dexamethasone caused 20% inhibition of DNA synthesis and a significant reduction in cell number. When cells were pretreated for 48h with dexamethasone and then received a 2h challenge with isoproterenol, the inhibitory effects on DNA synthesis were less than additive: the net effect on DNA synthesis was no greater than that of isoproterenol alone, and the effect on DNA content was not distinguishable from that seen with just the dexamethasone pretreatment. However, when both treatments were combined for 48h, the net effects on DNA synthesis and DNA content were augmented: decrements were greater than those achieved by either treatment alone.

In addition to measurements of DNA content, drug effects on the number of MDA-MB-231 cells were established by actual cell counts (Fig. 5). Sustained isoproterenol treatment reduced the total number of cells by over 20% and a comparable effect was seen for dexamethasone. Combined treatment with dexamethasone and isoproterenol had a comparably greater effect (30%), albeit not equivalent to the summation of the two individual effects. In order to maintain cAMP levels at the highest possible value, we also treated the cells with the phosphodiesterase inhibitor, theophylline, with or without isoproterenol (Fig. 5). Theophylline completely arrested mitosis, so that addition of isoproterenol had no further effect. The selectivity of the treatments towards mitosis were confirmed by examining cell morphology (Fig. 6). Isoproterenol and dexamethasone, alone or in combination, reduced the number of cells. Theophylline caused

massive reductions in cell number but the remaining cells were correspondingly larger than in the control group, indicating that the treatment did not prevent postmitotic cell growth.

To determine whether the effects of isoproterenol are shared by all cancer cells expressing β-adrenoceptors, we compared the effects on MDA-MB-231 cells with those of rat C6 glioma cells (Fig. 7). In contrast to the human breast cancer cells, C6 cells showed neither inhibition of DNA synthesis nor a reduction in DNA content over comparable periods.

The maintenance of isoproterenol-induced inhibition of DNA synthesis in MDA-MB-231 cells over a 48h span of continuous treatment suggested that either agonist-induced receptor downregulation or desensitization were not present in these cells, or alternatively, that stimulation of only a small number of receptors was sufficient to inhibit mitosis. Receptor downregulation can be selective for different subtypes and accordingly, we first evaluated which subtype was present in MDA-MB-231 cells. Using the β_1-selective antagonist, CGP20712A, we found that displacement of [^{125}I]iodopindolol involved a single class of sites displaying an IC50 in the μM range (Fig. 8). For contrast, we prepared cardiac cell membranes from one day old rats (Slotkin et al., 1994a), who display predominance of the β_1-subtype (Slotkin et al., 1994b): in this preparation, CGP20712A displayed two IC50 values, one in the nM range corresponding to the major cardiac receptor population, and a minor component which, like the MDA-MB-231 cells, displayed an IC50 in the μM range. Accordingly, the subtype expressed by MDA-MB-231 cells is almost exclusively β_2.

We next determined whether isoproterenol treatment of MDA-MB-231 cells causes β_2-receptor downregulation and/or uncoupling of the receptors from their ability to stimulate adenylyl cyclase. In untreated cells, adenylyl cyclase activity declined over 50% during the span of log-phase replication (Fig. 9). However, the adenylyl cyclase response to isoproterenol fell by a significantly smaller proportion than did any of the other measures and the concentration of β-receptors was maintained at the same level throughout replication and growth. Relative to total cyclase catalytic activity (Mn^{2+}), the isoproterenol response actually increased over the course of culturing. After 1 day in culture, isoproterenol evoked 75 ± 3% of the total catalytic response exemplified by Mn^{2+}, whereas after 3-4 days in culture, the two stimulations were indistinguishable: isoproterenol evoked 97 ± 3% of the total response ($p < 0.0001$ compared to the proportion after 1 day in culture).

Despite the fact that isoproterenol-induced inhibition of DNA synthesis was maintained throughout a 48h drug exposure, receptor downregulation was apparent immediately upon introduction of the drug (Fig. 10). A concentration-dependent reduction in receptor binding was evident within 1h, with nearly complete downregulation by 24h. Receptor binding then remained at 5-10% of control values throughout 72h of exposure. Isoproterenol concentrations as low as 1 nM produced significant, albeit submaximal, reductions in receptor binding after 24h of exposure. Scatchard analysis confirmed that the loss of receptor binding reflected a decrease in the number of receptors as measured by maximal binding, rather than a change in receptor affinity as monitored by the K_d. In additional studies, we found that a 2h daily isoproterenol exposure was sufficient to cause full receptor downregulation. We treated cells for three days in succession,

using 100 μM isoproterenol for 2h each day, followed in each case by 22h without drug; 22h after the third day's exposure, receptor binding was only 6 ± 1% of control values (n=6, $p < 0.0001$). Similarly, even when we reduced the concentration to 1 μM with exposure for 2h per day over a two day span, receptor measurements made 22h after the last exposure still indicated robust downregulation (8 ± 1% of control, n=6, $p < 0.0001$).

We also determined whether agonist-induced receptor downregulation was accompanied by loss of the adenylyl cyclase response to isoproterenol (Fig. 11). Treatment of cells with 1 μM isoproterenol for 2h, which caused approximately a 25% reduction in β-receptor binding, also elicited a comparable loss of the membrane response of adenylyl cyclase to isoproterenol. However, changes at the level of G-protein function were evident: basal enzyme activity, measured in the presence of GTP, also showed significant and immediate reductions, and the response to maximal G-protein activation by fluoride was impaired by a small amount. After a 48h exposure to isoproterenol, desensitization of the membrane adenylyl cyclase response to isoproterenol reached 75%, not quite as large as the degree of receptor downregulation; again, effects on basal adenylyl cyclase activity also were present, but were not as notable as the change in the β-receptor-mediated response. At no point did we note any decline in the expression or catalytic activity of adenylyl cyclase itself, as monitored by the effect of Mn^{2+}.

In light of the enhanced effect on cell replication of the combination of dexamethasone and isoproterenol treatment, we also examined their interaction at the levels of β-adrenoceptor binding and adenylyl cyclase activity. Pretreatment of cells with dexamethasone for 24h had no effect on β-receptor binding (Fig. 12, top panel). However, when the pretreatment was combined with a subsequent, 4h exposure to isoproterenol, it enhanced the downregulation caused by the receptor agonist. Dexamethasone had only small effects on adenylyl cyclase activity (Fig. 12, bottom panel). By itself, dexamethasone lowered the membrane response to isoproterenol by a few percent. When dexamethasone pretreatment was superimposed on short-term isoproterenol treatment of the cells, basal adenylyl cyclase activity was inhibited slightly less than with isoproterenol alone and the fluoride response was inhibited somewhat more. However, the agonist-induced desensitization of the specific response to isoproterenol was just as prominent after dexamethasone pretreatment as it was without pretreatment.

Year 2

In year 1, we found that replication of MDA-MB-231 human breast cancer cells, an estrogen-insensitive cell line, is inhibited by stimulation of β-adrenoceptors located on the cell membrane, and that the underlying mechanism is stimulation of adenylyl cyclase activity, resulting in increased intracellular cAMP levels. β-Adrenoceptors are expressed on a variety of cancer cell lines derived from epithelial or secretory tumors [7,27,29,33,37], including a number of breast cancers [15,31,36,37,48,55,56]. Nevertheless, there are also many cell lines that either do not express β-adrenoceptors, express only small concentrations of the receptors, or that respond to receptor stimulation by enhanced cell replication rather than inhibition [36,37,62]. In year 2, we explored mechanisms to raise intracellular cAMP levels that do not depend solely on the expression of β-adrenoceptors.

In year 1, we compared the effects of β-adrenoceptor agonists with membrane-permeable cAMP analogs and with theophylline, which inhibits phosphodiesterase, the enzyme that mediates cAMP breakdown. The potential anticancer actions of theophylline or related xanthines were proposed over a decade ago [61], and a number of studies have shown that theophylline reduces cell division in a number of different cancer cell lines [18,26,39]. Surprisingly, we found that theophylline had greater effects on mitosis and cell morphology in the MDA-MB-231 breast cancer line than did either a β-adrenoceptor agonist or cAMP analogs, suggesting extra effects of this xanthine derivative besides inhibition of phosphodiesterase. This view is reinforced by the dichotomy in the cAMP and theophylline responses of MCF-7 breast cancer cells, where cAMP enhances the mitotic response to prolactin, whereas theophylline blocks the response [40]. Indeed, although phosphodiesterase inhibitors, like theophylline, reduce cell replication via the increase in cAMP levels, they clearly affect cell differentiation through multiple mechanisms [20]. Theophylline has a number of important cellular actions other than phosphodiesterase inhibition, notably its activity toward adenosine receptors, and its ability to generate reactive oxygen species. Ordinarily, adenosine receptor stimulation is thought to enhance cancer cell replication by decreasing intracellular cAMP levels [25]; however, theophylline and its derivatives possess a mixture of adenosine agonist-antagonist properties that can contribute to antitumor activity, but that depend on the differentiation state of the target cells [5]. Since theophylline is a xanthine derivative, there is the further possibility of free radical generation via xanthine oxidase, with the formation of cytotoxic levels of nitric oxide and peroxynitrite [58]. Free radicals produced by xanthine oxidase also affect cancer cell adhesion [51] and elicit DNA strand breaks [30,63].

In year 2, we evaluated the effects of theophylline on MDA-MB-231 human breast cancer cells, concentrating on comparisons of antimitotic activity, cytotoxicity and cell adhesion characteristics, and have contrasted the effects of theophylline with 3-isobutyl-1-methylxanthine (IBMX), a derivative that also inhibits phosphodiesterase and generates free radicals, but that is relatively insensitive toward adenosine receptors [58].

Methods

Except for the specific drug treatments, conditions for cell cultures and outcomes were identical to those in year 1.

Results

As found previously [48], MDA-MB-231 cells grew in log-phase from 1 to 4 days after plating. The doubling time, based on cell counts and DNA content, was approximately 3 days, whereas the doubling of confluence was much quicker (1.5 days), indicating growth both by cell enlargement and replication (data not shown). Accordingly, drug treatments were initiated after one day in culture and were terminated at various times during the log-phase of growth.

Introduction of theophylline into the culture medium caused an immediate, concentration-dependent reduction in [^{3}H]thymidine incorporation into DNA (Fig. 12). Significant inhibition

was seen at 1 mM theophylline and inhibition was >50% at 10 mM. After extended exposure (24h), there was no change in the concentration threshold for inhibition of DNA synthesis but the effect at 10 mM increased to >95% inhibition. Whereas there were no immediate effects of theophylline on cell number as monitored by DNA content, levels were 25% subnormal by 24h after beginning exposure to the highest theophylline concentration; in an additional experiment conducted at 72h, the DNA content in the theophylline-treated cells was reduced even further, to only 17 ± 1% of the control values ($p < 0.0001$, n=10 for each group).

To determine whether the effects of theophylline on macromolecule synthesis were specific for DNA, we next determined whether similar treatment would affect protein synthesis (Fig. 13). Twenty-four hours after beginning exposure to theophylline, there was a significant decrement in protein synthesis, with a lower threshold than had been seen for DNA synthesis: deficits were significant with as little as 0.1 mM theophylline. However, the maximal effect seen with 10 mM theophylline was not as great as for DNA synthesis: protein synthesis was reduced to 40% of control values, whereas inhibition of DNA synthesis was nearly total at that concentration and time. After 48h of exposure to 10 mM theophylline, cell membrane protein was reduced by 50% (control, 0.62 ± 0.02 mg/dish; theophylline, 0.30 ± 0.01, $p < 0.0001$; n=6).

The differences in the concentration-response curves for inhibition of DNA and protein synthesis suggested that theophylline might have additional effects besides antimitotic activity. Accordingly, we assessed cell number and viability after a 48h exposure to 10 mM theophylline (Fig. 14). Theophylline evoked nearly a 60% reduction in the total number of cells. Over and above the deficit in cell number, theophylline had adverse effects on viability of the remaining cells, as assessed by two indices, detachment and exclusion of trypan blue. The proportion of cells in each dish that were detached from the surface of the culture was nearly tripled. In addition, there was a doubling of the proportion of total cells (attached and detached) stained with trypan blue. Theophylline-induced cell detachment loss and of viability were related to each other. In control cultures, 5.3 ± 0.3% of the attached cells failed to exclude trypan blue, whereas 36 ± 4% of the detached cells were nonviable ($p < 0.0001$ versus attached cells). In the theophylline group, both these values were higher: 9.8 ± 0.7% nonviable attached cells ($p < 0.0001$ versus control group) and 55 ± 2% nonviable detached cells ($p < 0.0001$ versus control group; $p < 0.0002$ versus theophylline-exposed attached cells). Thus, over half of the detached cells in the theophylline group were nonviable, whereas only a third of those in the control group were nonviable.

Theophylline possesses distinctly different pharmacological modalities, as a phosphodiesterase inhibitor, an adenosine receptor antagonist, and a xanthine. Therefore we conducted similar studies using IBMX, a phosphodiesterase inhibitor with much lower activity toward adenosine receptors. Because IBMX requires a solvent (0.1% DMSO) in order to dissolve in an aqueous medium, we compared untreated cells to cells exposed to DMSO alone, and to cells exposed to IBMX in DMSO vehicle (Fig. 15). With a 24h exposure, 1 mM IBMX evoked 80% inhibition of DNA synthesis, a much greater reduction ($p < 0.0001$) than that seen at the same concentration of theophylline (≈15% inhibition). Nevertheless, IBMX elicited only a 10% reduction in DNA content, no greater than that seen with the much less effective theophylline treatment. Indeed,

when compared to 10 mM theophylline, a concentration that produced roughly equivalent inhibition of DNA synthesis to that caused by 1 mM IBMX, the effect of IBMX on DNA content was significantly less: 9 ± 3% reduction for IBMX (Fig. 15), 26 ± 1% reduction for theophylline (Fig. 12; $p < 0.0001$ versus IBMX).

In our previous work [48], we showed that stimulation of cellular cAMP levels with a β-adrenoceptor agonist led to reductions in DNA synthesis, accompanied by desensitization at the level of receptor-mediated adenylyl cyclase activity. Accordingly, we evaluated whether theophylline, which inhibits cAMP breakdown, also targets the function of this signaling cascade. After a 48h exposure to 10 mM theophylline, basal adenylyl cyclase activity was significantly reduced but signaling mediated by the β-adrenoceptor stimulant, isoproterenol, was maintained (Fig. 16). Similarly, the response to fluoride, which evokes maximal stimulation of the G-proteins that link the receptors to adenylyl cyclase, was unaffected despite the lowering of basal enzyme activity. The response to manganese, which stimulates adenylyl cyclase directly, without the requirement for receptors or G-proteins, was significantly enhanced by theophylline treatment.

Year 3

In our work for years 1 and 2 [45,48], we found that theophylline arrests mitosis and elicits cytotoxicity through mechanisms over and above inhibition of phosphodiesterase, the enzyme that mediates cyclic AMP breakdown. Theophylline has antitumor activity in a number of different cancer lines [18,26,39,61] and in many cases there is a dichotomy between the responses to theophylline *versus* cyclic AMP [20,40,45].

Xanthine derivatives exhibit activity toward adenosine receptors as well as toward generation of reactive oxygen species (ROS) [5,30,51,58,63]. Accordingly, in the third year, we evaluated the ability of theophylline and 3-isobutyl-1-methylxanthine (IBMX), a derivative that also inhibits phosphodiesterase and generates free radicals [58], to generate ROS in MDA-MB-231 cells.

Methods

Cell cultures and treatments were the same as described for years 1 and 2.

Assay conditions were carried out as described in earlier work with PC12 cells [13]. Cells were harvested in Krebs-Ringer-bicarbonate buffer (120 mM NaCl; 5 mM KCl; 1 mM $MgSO_4$; 1 mM $CaCl_2$; 10 mM HEPES; 25 mM $NaHCO_3$; 1 mM NaH_2PO_4; 10 mM glucose) and sedimented at $250 \times g$ for 10 minutes. The supernatant solution was removed and the remaining cells were washed twice with buffer and resedimented. The cells were counted and the cell concentration was adjusted to 6.5×10^5 per mL. An aliquot of cell suspension was loaded with 30 μM dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR) for 15 minutes in the dark, and then fluorescence was monitored for the ensuing 10 min with or without addition of 1 mM theophylline or IBMX. Quantitation was based on the steady-state slope of fluorescence achieved by the end of the 10 min test period.

In addition to determining whether theophylline or IBMX lead to ROS generation, we also evaluated whether the drugs potentiated the oxidative response to 0.3 mM sodium nitroprusside (SNP), a compound that generates ROS directly and also through intracellular production of nitric oxide [13,21]. SNP was added simultaneously with the drugs [13].

Results

Without addition of drugs, there was a small, spontaneous rate of oxidation of the intracellular dye; incubation with SNP produced a large increase in the generation of oxidative species (Fig. 17). Addition of 1 mM theophylline caused a significant increase in ROS generation and also increased the response to SNP by the same proportion. An equimolar concentration of IBMX caused a much greater increase in ROS generation and in the SNP response.

Figure Legends

Fig. 1. Effects of isoproterenol treatment on DNA synthesis and content, presented as the percentage change from control values. Data represent means and standard errors obtained from 12-96 determinations for each concentration and time point. Isoproterenol was added for 2h or 48h, with inclusion of [^{3}H]thymidine for the final hour. ANOVA across all treatments appears at the top of each panel and asterisks denote individual treatments that differ significantly from the control.

Fig. 2. Effects of a 2h treatment with isoproterenol (Iso), 8-bromo-cAMP (8Br), or propranolol (Pro) treatment on DNA synthesis, presented as the percentage change from control values. Data represent means and standard errors obtained from 10-26 determinations for each concentration and time point. ANOVA across all treatments appears at the top of each panel; asterisks denote individual treatments that differ significantly from the control and the dagger denotes a significant difference between Pro + Iso and Iso alone. In addition, two-factor ANOVA for the effects of propranolol on the isoproterenol response indicated complete blockade ($p < 0.002$ for the main effect of isoproterenol, $p < 0.02$ for the main effect of propranolol, $p < 0.05$ for the interaction of the two treatments). None of the treatments produced a significant change in DNA content (data not shown).

Fig. 3. Effects of dexamethasone (Dex) alone or in combination with isoproterenol (Iso), on DNA synthesis and content, presented as the percentage change from control values. Data represent means and standard errors obtained from 11-54 determinations for each treatment. ANOVA across all treatments appears at the top of each panel; asterisks denote individual treatments that differ significantly from the control and the daggers denote significant differences between Dex 48h + Iso 48h, and the corresponding treatments with Dex or Iso alone. In addition, for DNA synthesis, two-factor ANOVA (dexamethasone 48h × isoproterenol 2h) indicates significant main effects of dexamethasone ($p < 0.0005$) and isoproterenol ($p < 0.0001$) but no interaction between the two treatments; with 48h of both dexamethasone and isoproterenol treatment, there were significant main effects of both treatments ($p < 0.0001$ for each) as well as a significant interaction of dexamethasone × isoproterenol ($p < 0.002$). For DNA content, two-factor ANOVA (dexamethasone 48h × isoproterenol 2h) indicates a significant main effect of

dexamethasone ($p < 0.009$) but no effect of isoproterenol; with 48h of both dexamethasone and isoproterenol treatment, there were significant main effects of both treatments ($p < 0.0001$ for each).

Fig. 4. Effects of a 72h treatment with isoproterenol (Iso), dexamethasone (Dex), or theophylline (Theo) on cell number, presented as the percentage change from control values. Data represent means and standard errors obtained from 10-22 determinations for each treatment. ANOVA across all treatments appears at the top of each panel. Asterisks denote individual treatments that differ significantly from the control; the dagger denotes a significant difference between Dex + Iso and either treatment alone; § denotes significant differences between theophylline with or without isoproterenol, as compared to all other treatments. In addition, two-factor ANOVA across the dexamethasone and isoproterenol treatments indicates significant main effects of each treatment alone ($p < 0.0001$) as well as a significant interaction of dexamethasone × isoproterenol ($p < 0.03$). Across the theophylline and isoproterenol groups, there were significant main effects of each treatment alone ($p < 0.0001$) as well as a significant interaction of theophylline × isoproterenol ($p < 0.0001$).

Fig. 5. Phase-contrast microscopic appearance of MDA-MB-231 cells after a 72h treatment with isoproterenol, dexamethasone, or theophylline. Scale bar appears in the upper left panel.

Fig. 6. Effects of 10 μM isoproterenol (Iso) on DNA synthesis and content in rat C6 glioma cells, presented as the percentage change from control values. Data represent means and standard errors obtained from 11-30 determinations for each time point. ANOVA across all treatments appears within each panel.

Fig. 7. Binding of [^{125}I]iodopindolol to β-adrenoceptors in cell membranes prepared from MDA-MB-231 cells and from one day old rat heart, and its displacement by the β_1-specific antagonist, CGP20712A. Each data point represents an individual determination, shown as the percentage of values obtained in the absence of displacer. The [^{125}I]iodopindolol concentration was 67 pM.

Fig. 8. Adenylyl cyclase activity and β-adrenoceptor binding in MDA-MB-231 cells during cell replication and growth in culture. Cells were cultured for the indicated time periods and then membranes were isolated and enzyme activity determined under basal conditions or in the presence of 100 μM isoproterenol (Iso), 10 mM fluoride (F^-) or 10 mM Mn^{2+}. Data represent means and standard errors obtained from 6-24 determinations for each measurement at each time point. ANOVA across both time points and all stimulants appears within the panel and asterisks denote measures for which the values after 3-4 days in culture are significantly lower than the initial values. In addition, the cyclase response to isoproterenol declines less than the response to other stimulants ($p < 0.0001$).

Fig. 9. β-Adrenoceptor downregulation caused by isoproterenol treatment. Data represent means and standard errors obtained from the number of determinations shown in parentheses, determined as the percentage change from control values. In the top and middle panels, ANOVA across all time points or treatments appears at the top and asterisks denote individual values that

differ significantly from the control. In the bottom panel, ANCOVA appears for the overall differences between Scatchard plots in control and isoproterenol-treated (Iso) cells.

Fig. 10. Desensitization of adenylyl cyclase evoked by exposure to 1 μM isoproterenol. Cells were treated with isoproterenol for the indicated time period and then membranes were isolated and enzyme activity determined under basal conditions or in the presence of 100 μM isoproterenol (Iso), 10 mM fluoride (F^-) or 10 mM Mn^{2+}. Data represent means and standard errors obtained from 6-12 determinations at each time point, determined as the percentage change from control values. ANOVA across all stimulants and both time points appears at the top and asterisks denote individual values that differ significantly from the control. Two-factor ANOVAs (treatment × time) were also assessed for each variable. Across both time points, basal activity was significantly reduced ($p < 0.0001$ for the main effect of isoproterenol), the response to isoproterenol was reduced in a time-dependent fashion ($p < 0.0001$ for the main effect, $p < 0.0001$ for the treatment × time interaction), the response to fluoride was reduced ($p < 0.0001$ for the main effect), and the response to Mn^{2+} was unaffected.

Fig. 11. Effects of dexamethasone (Dex) alone or in combination with isoproterenol (Iso), on β-adrenoceptor binding (top) and adenylyl cyclase activity (bottom). Cells were pretreated with 10 μM dexamethasone for 24h (top) or 48h (bottom) and were then exposed to 1 μM isoproterenol for 4h. For adenylyl cyclase, enzyme activity was determined under basal conditions or in the presence of 100 μM isoproterenol (Iso), 10 mM fluoride (F^-) or 10 mM Mn^{2+}. Data represent means and standard errors obtained from 6-12 determinations for each treatment. For receptor binding, ANOVA across all treatments appears at the top; for adenylyl cyclase, ANOVA across all treatments and *in vitro* stimulants appears at the top, and lower order ANOVAs are shown for *in vitro* stimulant with each cluster of bars. Asterisks denote individual treatments that differ significantly from the control and the daggers denotes significant difference of combined treatment from the effects of dexamethasone or isoproterenol alone.

Fig. 12. DNA synthesis and content after 2 or 24h of theophylline exposure. Data represent means and standard errors obtained from 7-16 determinations at each concentration and time. Control [^{3}H]thymidine incorporation averaged 6000 dpm/μg DNA at 2h and 5100 at 24h; DNA content averaged 26 and 33 μg/dish, respectively. ANOVA across all concentrations and both time periods appears at the top of each panel. Asterisks denote individual values that differ from the corresponding control; in addition, the values at 10 mM theophylline are significantly different from those at 1 mM ($p < 0.0001$).

Fig. 13. Effects of 10 mM theophylline on protein synthesis, measured after a 24h exposure. Data represent means and standard errors obtained from 8 determinations at each concentration. Control [^{3}H]leucine incorporation averaged 700 dpm/μg DNA. ANOVA across all concentrations appears at the top of the panel. Asterisks denote individual values that differ from the corresponding control; in addition, the values for each theophylline concentration are significantly different from each other ($p < 0.02$ or better).

Fig. 14. Cell number and viability after prolonged theophylline exposure. Data represent means and standard errors obtained from 16 determinations. Viability was determined as the percentage of total cells (attached and detached) that failed to exclude trypan blue. Detached cells were determined as the proportion of total cells (viable and nonviable) floating in the medium.

Fig. 15. DNA synthesis and content after exposure to 1 mM IBMX. Data represent means and standard errors obtained from 10 determinations for each condition. Because 0.1% DMSO vehicle was required to dissolve IBMX, values are compared for untreated cells (control), vehicle, and IBMX. ANOVA across all conditions appears at the top of the panel, and asterisks denote individual values that differ from the corresponding control or DMSO values.

Fig. 16. Effects of exposure to 10 mM theophylline on adenylyl cyclase activity measured under basal conditions, or with addition of 100 μM isoproterenol, 10 mM sodium fluoride, or 10 mM manganese chloride. Data represent means and standard errors obtained from 6 determinations for each stimulant. Control activities were: basal, 27 pmol/min per mg protein; isoproterenol, 70; fluoride, 59; manganese, 73. ANOVA across all measures appears at the top of the panel and asterisks denote individual values that differ from the corresponding control.

Fig. 17. Generation of reactive oxygen species (ROS) in MDA-MB-231 cells. Cells were preloaded with dye and were then exposed to 1 mM theophylline or 3-isobutyl-1-methylxanthine (IBMX) for 10 min, either by themselves or concurrently with 0.3 mM sodium nitroprusside (SNP) challenge. The top panels show representative fluorescence determinations; note the different scale for samples to which SNP was added (right). The bottom panel shows the percent change from control values as means and standard errors, with the number of determinations shown in parentheses. ANOVA across all treatments appears within the panel and asterisks denote individual values that differ significantly from the corresponding control. In addition, the effects of the drugs are significant when assessed separately with or without SNP ($p < 0.0001$ in both cases) and the effects of IBMX are significantly greater than those of theophylline ($p < 0.0001$ either with or without SNP).

Fig. 1

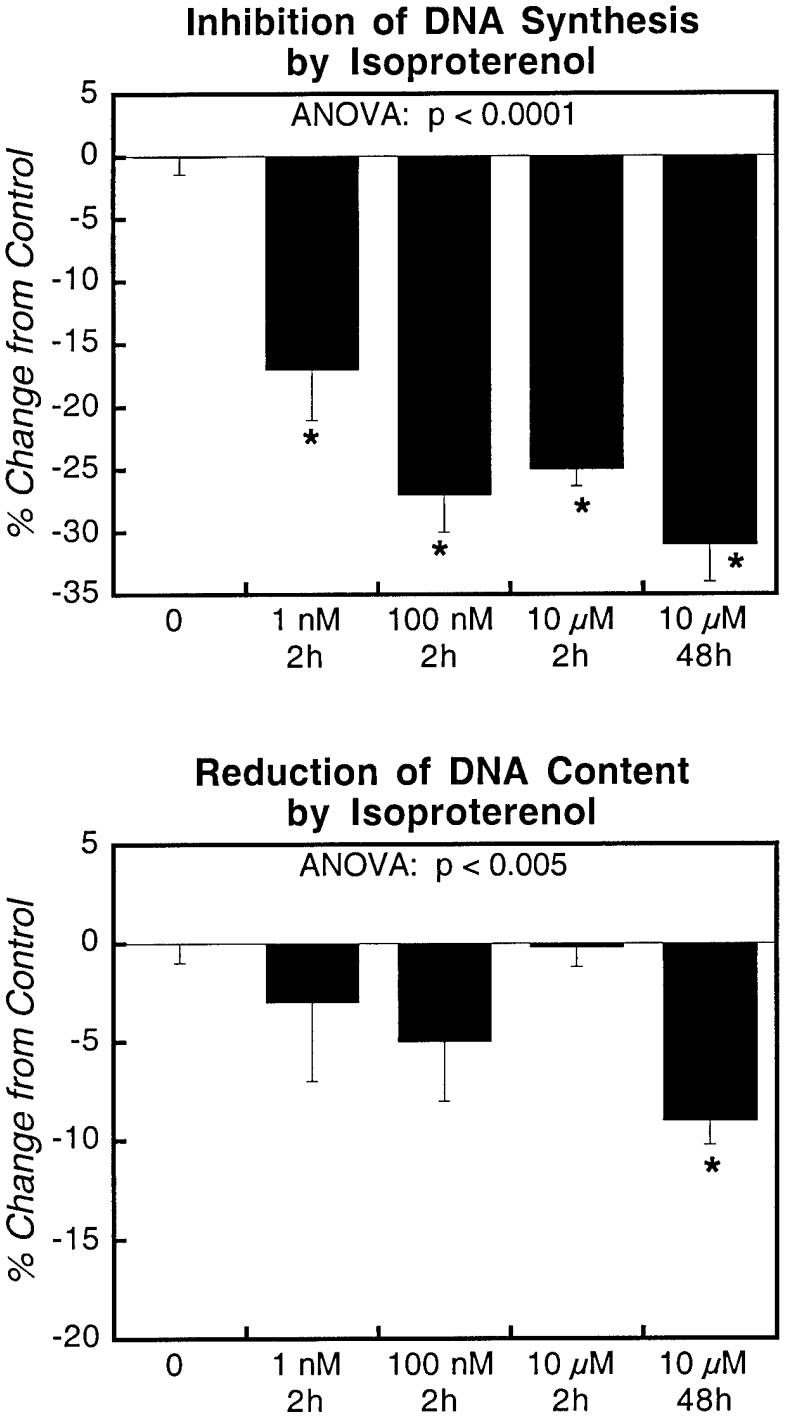

Inhibition of DNA Synthesis
by Isoproterenol
ANOVA: p < 0.0001
% Change from Control
5
0
-5
-10
-15
-20
-25
-30
-35
*
0
1 nM
2h
100 nM
2h
10 μM
2h
10 μM
48h
Reduction of DNA Content
by Isoproterenol
ANOVA: p < 0.005
% Change from Control
5
0
-5
-10
-15
-20
*
0
1 nM
2h
100 nM
2h
10 μM
2h
10 μM
48h

Fig. 2

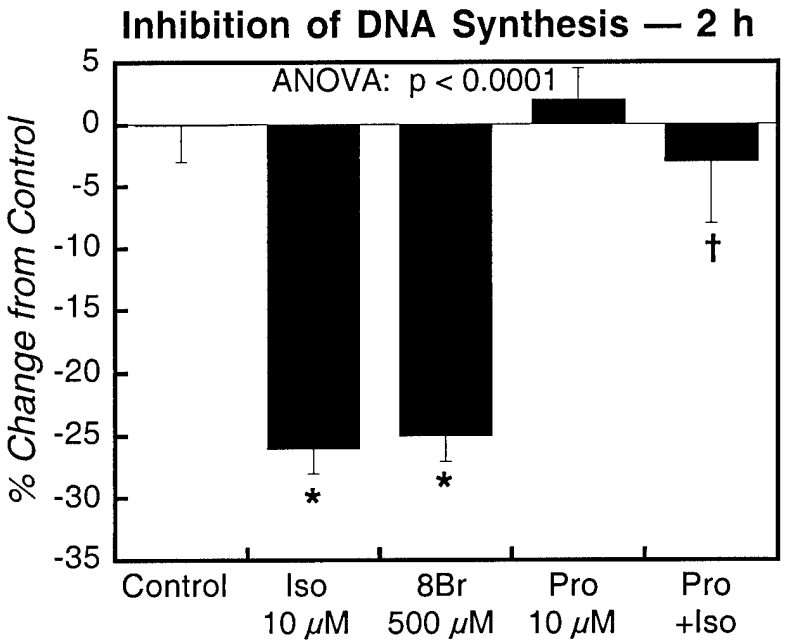

Inhibition of DNA Synthesis — 2 h
ANOVA: p < 0.0001
% Change from Control
5
0
-5
-10
-15
-20
-25
-30
-35
*
*
†
Control
Iso 10 μM
8Br 500 μM
Pro 10 μM
Pro +Iso

Fig. 3

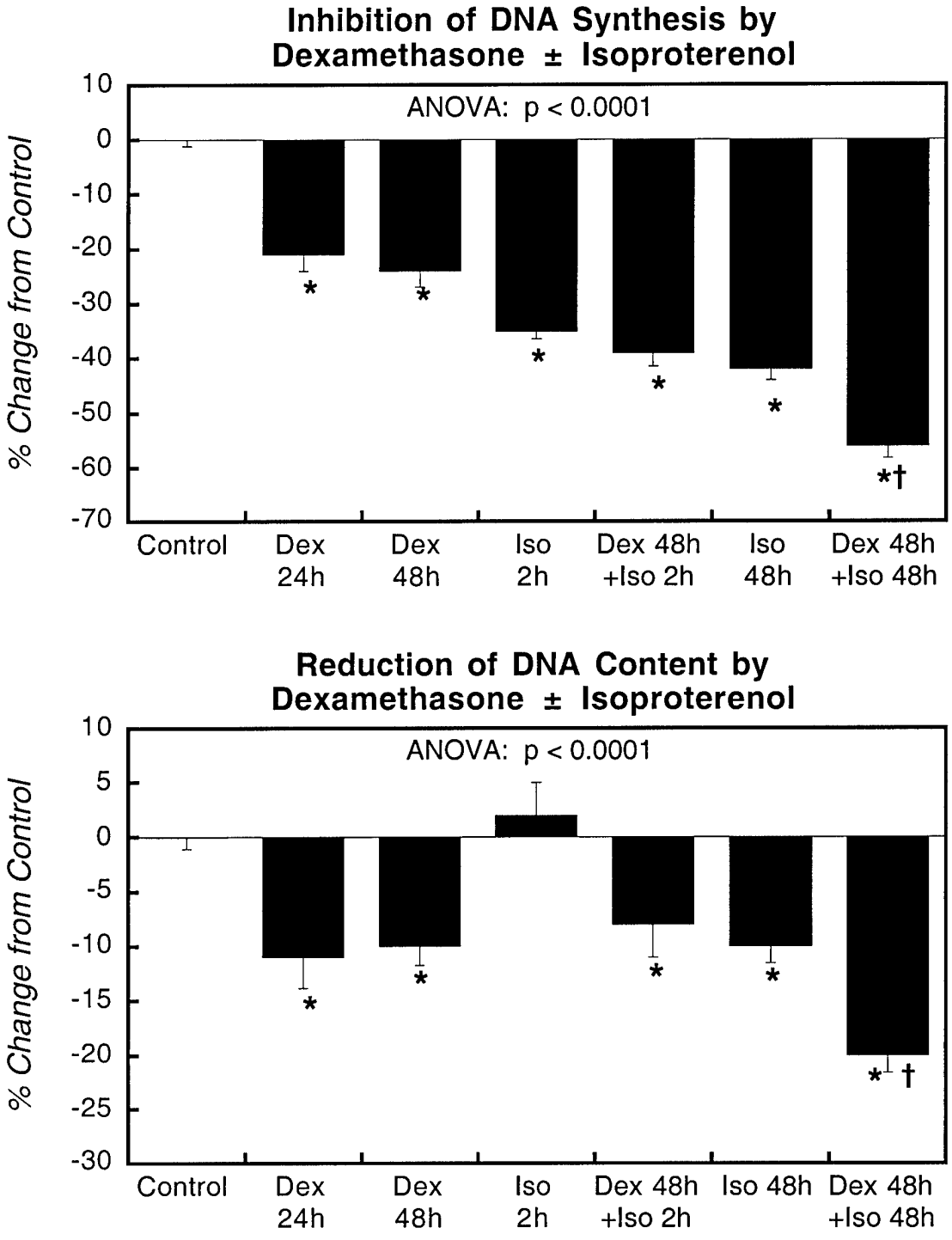

Inhibition of DNA Synthesis by
Dexamethasone ± Isoproterenol
ANOVA: p < 0.0001
% Change from Control
10
0
-10
-20
-30
-40
-50
-60
-70
Control
Dex 24h
Dex 48h
Iso 2h
Dex 48h +Iso 2h
Iso 48h
Dex 48h +Iso 48h
Reduction of DNA Content by
Dexamethasone ± Isoproterenol
ANOVA: p < 0.0001
% Change from Control
10
5
0
-5
-10
-15
-20
-25
-30
Control
Dex 24h
Dex 48h
Iso 2h
Dex 48h +Iso 2h
Iso 48h
Dex 48h +Iso 48h

Fig. 4

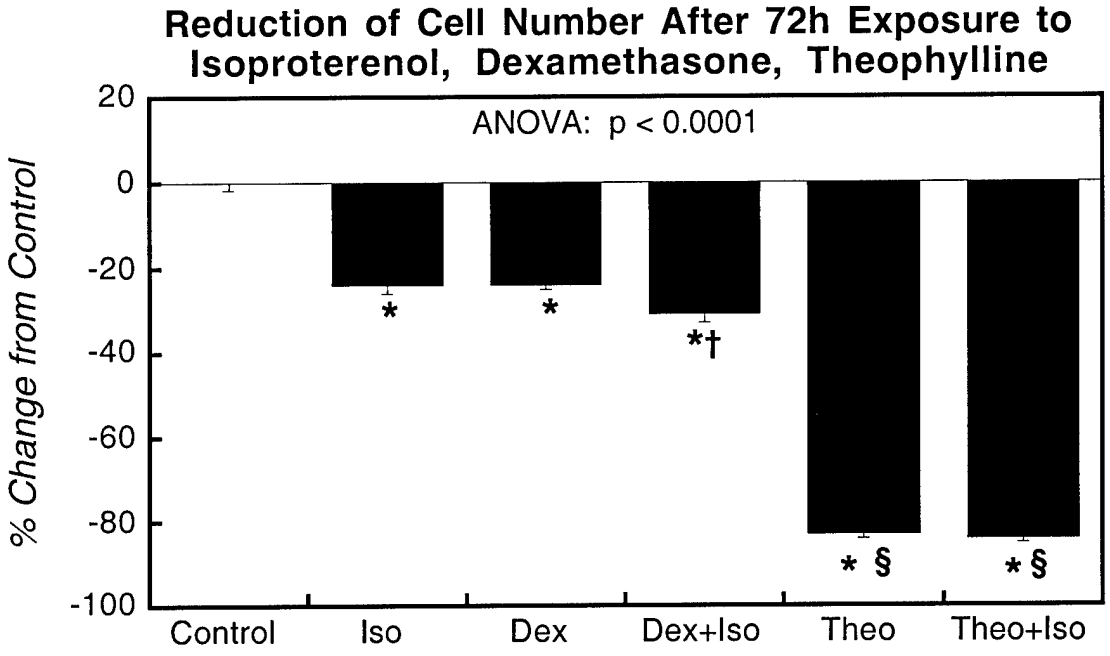

Reduction of Cell Number After 72h Exposure to Isoproterenol, Dexamethasone, Theophylline
ANOVA: p < 0.0001
% Change from Control
20
0
-20
-40
-60
-80
-100
*
*
*†
* §
* §
Control
Iso
Dex
Dex+Iso
Theo
Theo+Iso

Fig. 5

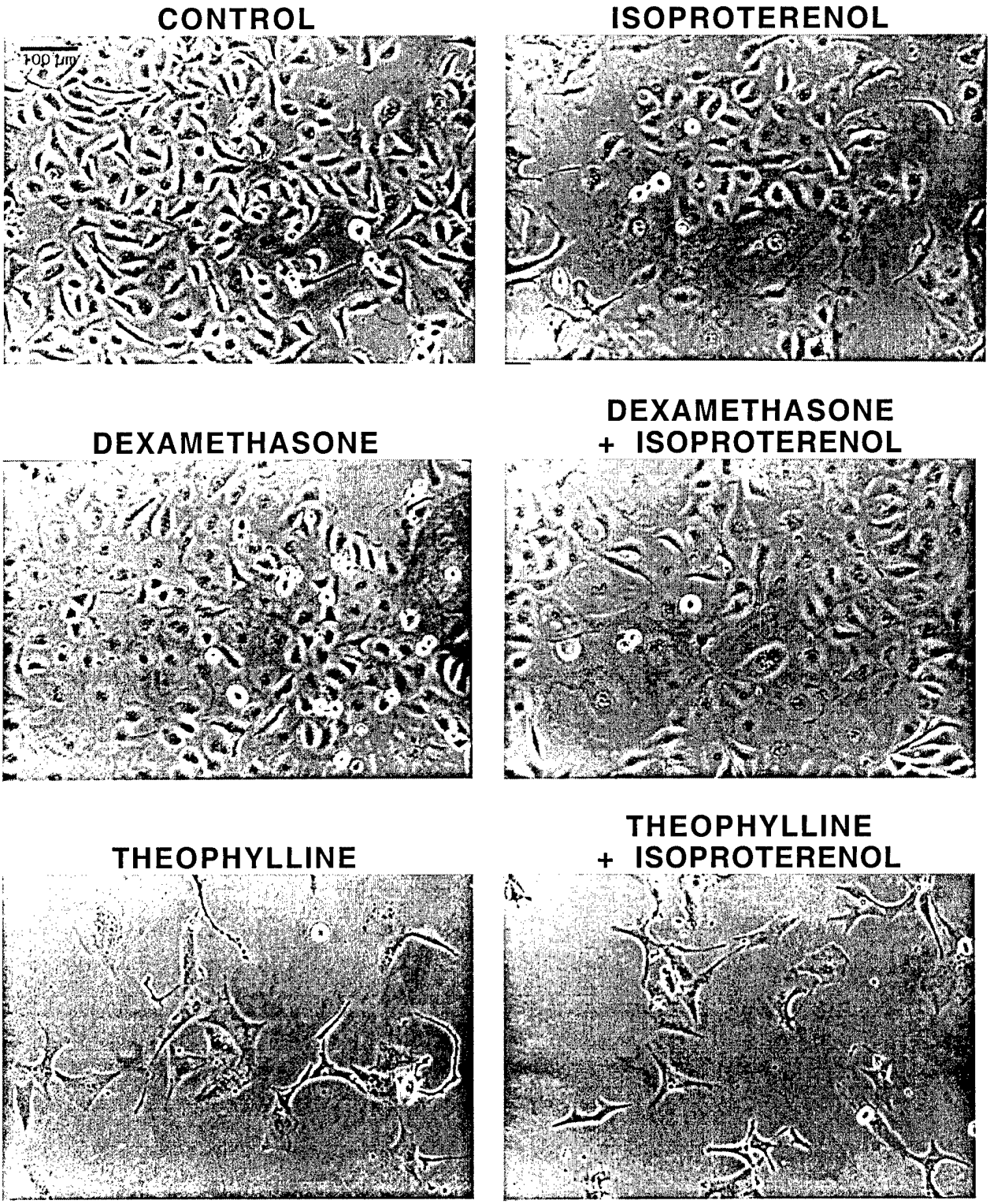

CONTROL
ISOPROTERENOL
DEXAMETHASONE
DEXAMETHASONE
+ ISOPROTERENOL
THEOPHYLLINE
THEOPHYLLINE
+ ISOPROTERENOL

Fig. 6

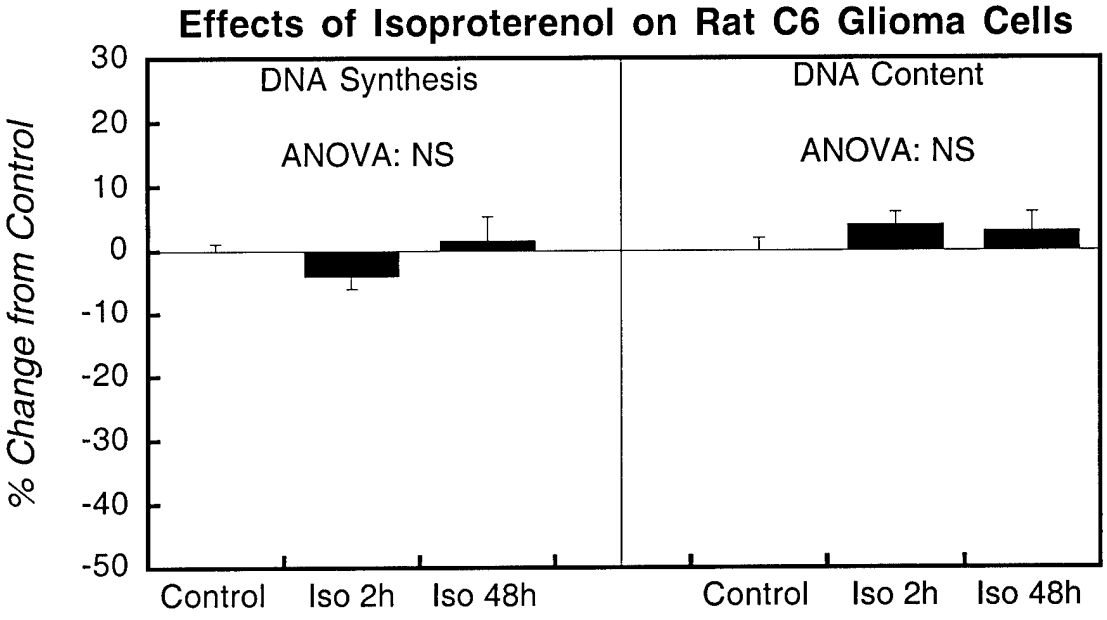

Effects of Isoproterenol on Rat C6 Glioma Cells
% Change from Control
30
20
10
0
-10
-20
-30
-40
-50
DNA Synthesis
ANOVA: NS
DNA Content
ANOVA: NS
Control
Iso 2h
Iso 48h
Control
Iso 2h
Iso 48h

Fig. 7

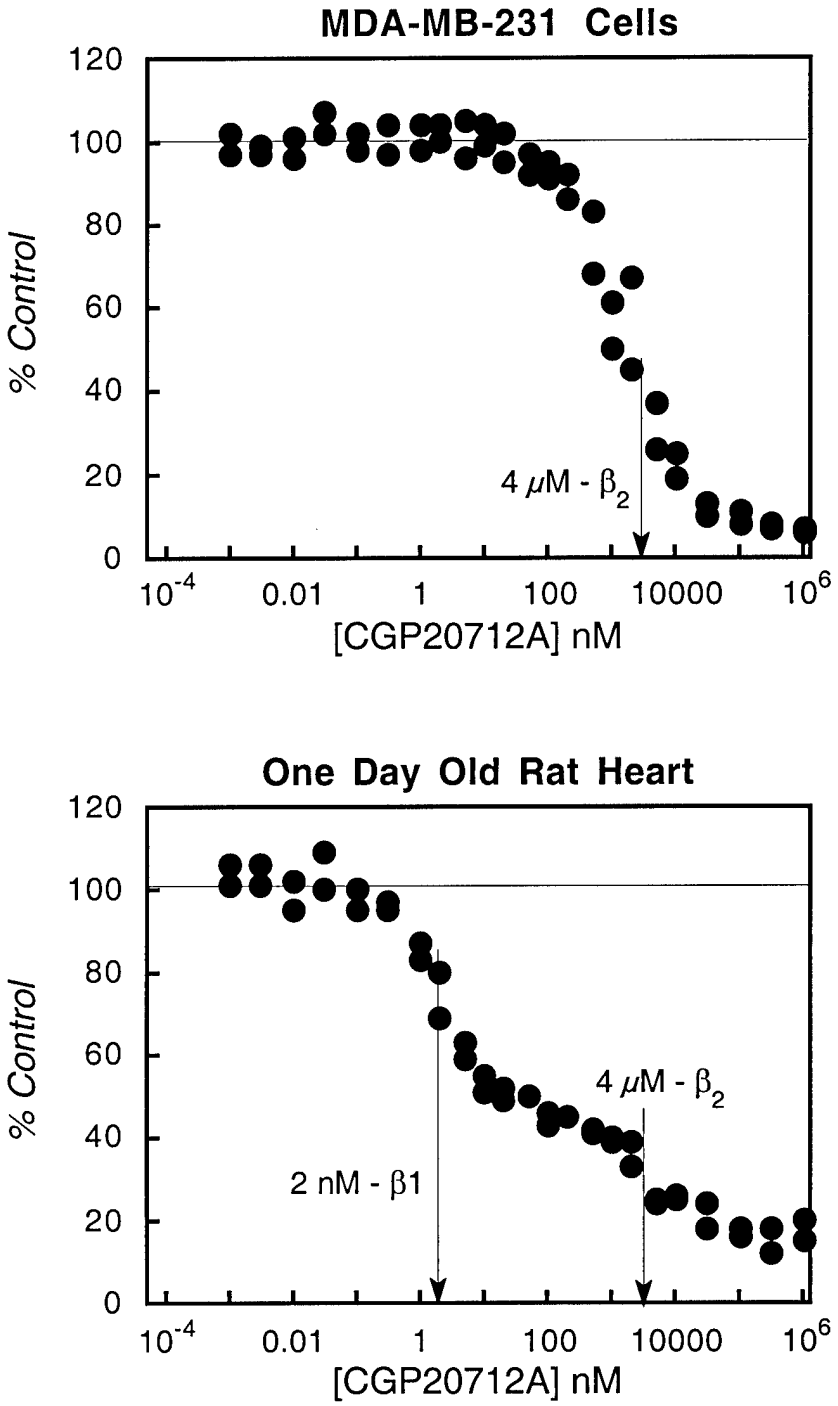

MDA-MB-231 Cells
% Control
120
100
80
60
40
20
0
4 μM - β2
10-4
0.01
1
100
10000
10^6
[CGP20712A] nM
One Day Old Rat Heart
% Control
120
100
80
60
40
20
0
4 μM - β2
2 nM - β1
10-4
0.01
1
100
10000
10^6
[CGP20712A] nM

Fig. 8

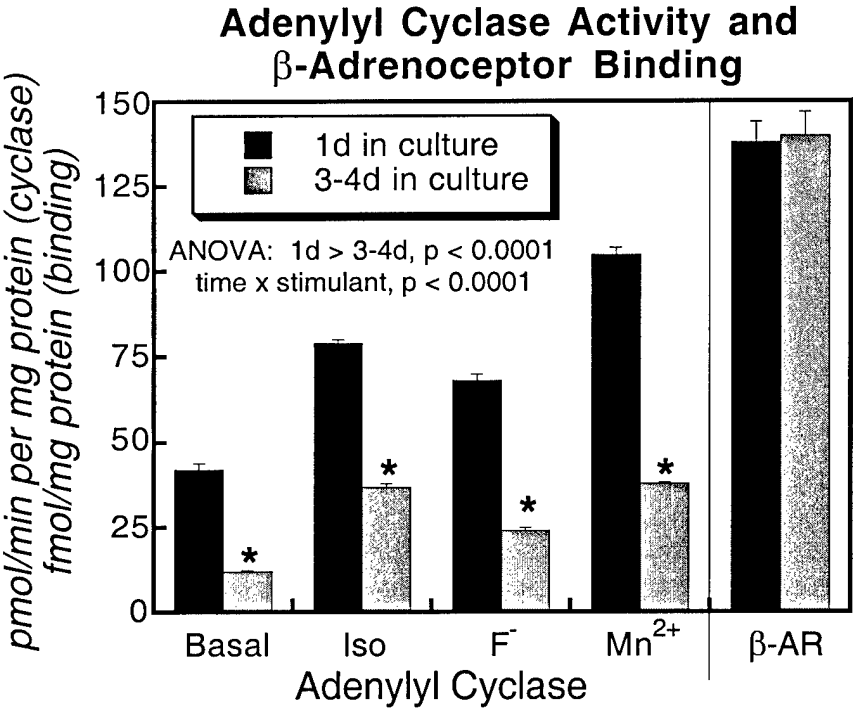

Adenylyl Cyclase Activity and
β-Adrenoceptor Binding
pmol/min per mg protein (cyclase)
fmol/mg protein (binding)
150
125
100
75
50
25
0
1d in culture
3-4d in culture
ANOVA: 1d > 3-4d, p < 0.0001
time x stimulant, p < 0.0001
*
Basal
Iso
F-
Mn2+
β-AR
Adenylyl Cyclase

Fig. 9

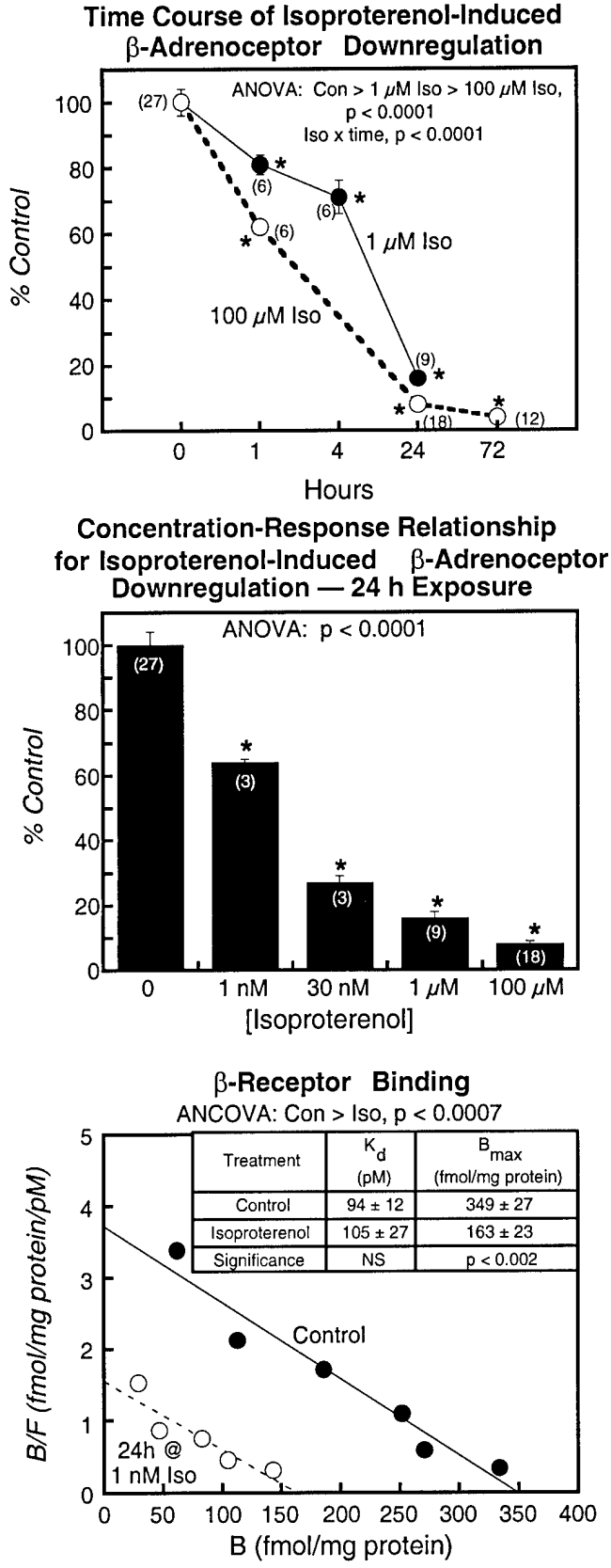

Time Course of Isoproterenol-Induced
β-Adrenoceptor Downregulation
ANOVA: Con > 1 μM Iso > 100 μM Iso,
p < 0.0001
Iso x time, p < 0.0001
% Control
Hours
1 μM Iso
100 μM Iso
Concentration-Response Relationship
for Isoproterenol-Induced β-Adrenoceptor
Downregulation — 24 h Exposure
ANOVA: p < 0.0001
[Isoproterenol]
β-Receptor Binding
ANCOVA: Con > Iso, p < 0.0007
Treatment
Kd (pM)
Bmax (fmol/mg protein)
Control
94 ± 12
349 ± 27
Isoproterenol
105 ± 27
163 ± 23
Significance
NS
p < 0.002
B/F (fmol/mg protein/pM)
B (fmol/mg protein)
24h @
1 nM Iso

Fig. 10

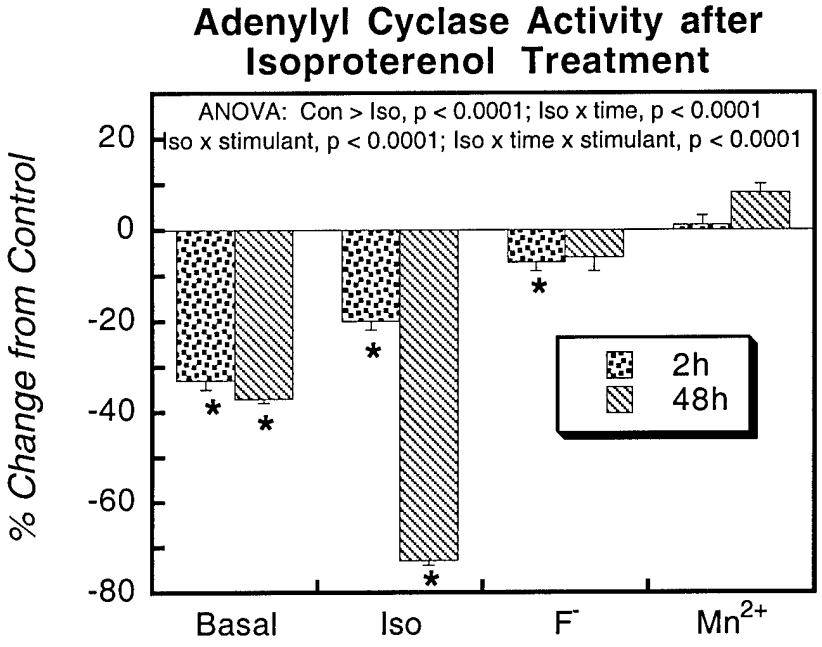

Adenylyl Cyclase Activity after Isoproterenol Treatment
ANOVA: Con > Iso, p < 0.0001; Iso x time, p < 0.0001
Iso x stimulant, p < 0.0001; Iso x time x stimulant, p < 0.0001
% Change from Control
20
0
-20
-40
-60
-80
2h
48h
Basal
Iso
F-
Mn2+

Fig. 11

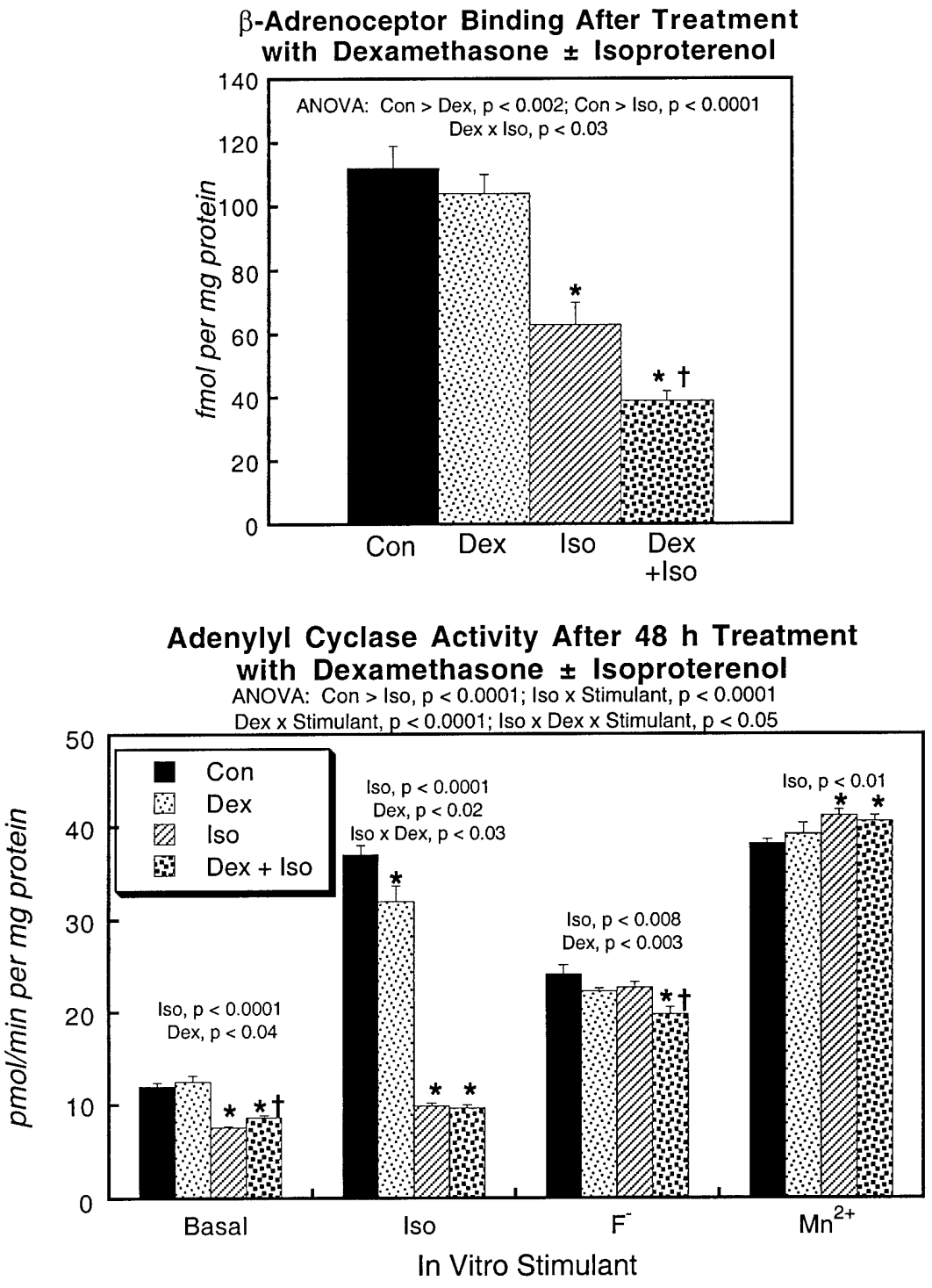

β-Adrenoceptor Binding After Treatment with Dexamethasone ± Isoproterenol
ANOVA: Con > Dex, p < 0.002; Con > Iso, p < 0.0001
Dex x Iso, p < 0.03
fmol per mg protein
140
120
100
80
60
40
20
0
Con
Dex
Iso
Dex +Iso
Adenylyl Cyclase Activity After 48 h Treatment with Dexamethasone ± Isoproterenol
ANOVA: Con > Iso, p < 0.0001; Iso x Stimulant, p < 0.0001
Dex x Stimulant, p < 0.0001; Iso x Dex x Stimulant, p < 0.05
pmol/min per mg protein
50
40
30
20
10
0
Con
Dex
Iso
Dex + Iso
Iso, p < 0.0001
Dex, p < 0.04
Iso, p < 0.0001
Dex, p < 0.02
Iso x Dex, p < 0.03
Iso, p < 0.008
Dex, p < 0.003
Iso, p < 0.01
Basal
Iso
F-
Mn2+
In Vitro Stimulant

Fig. 12

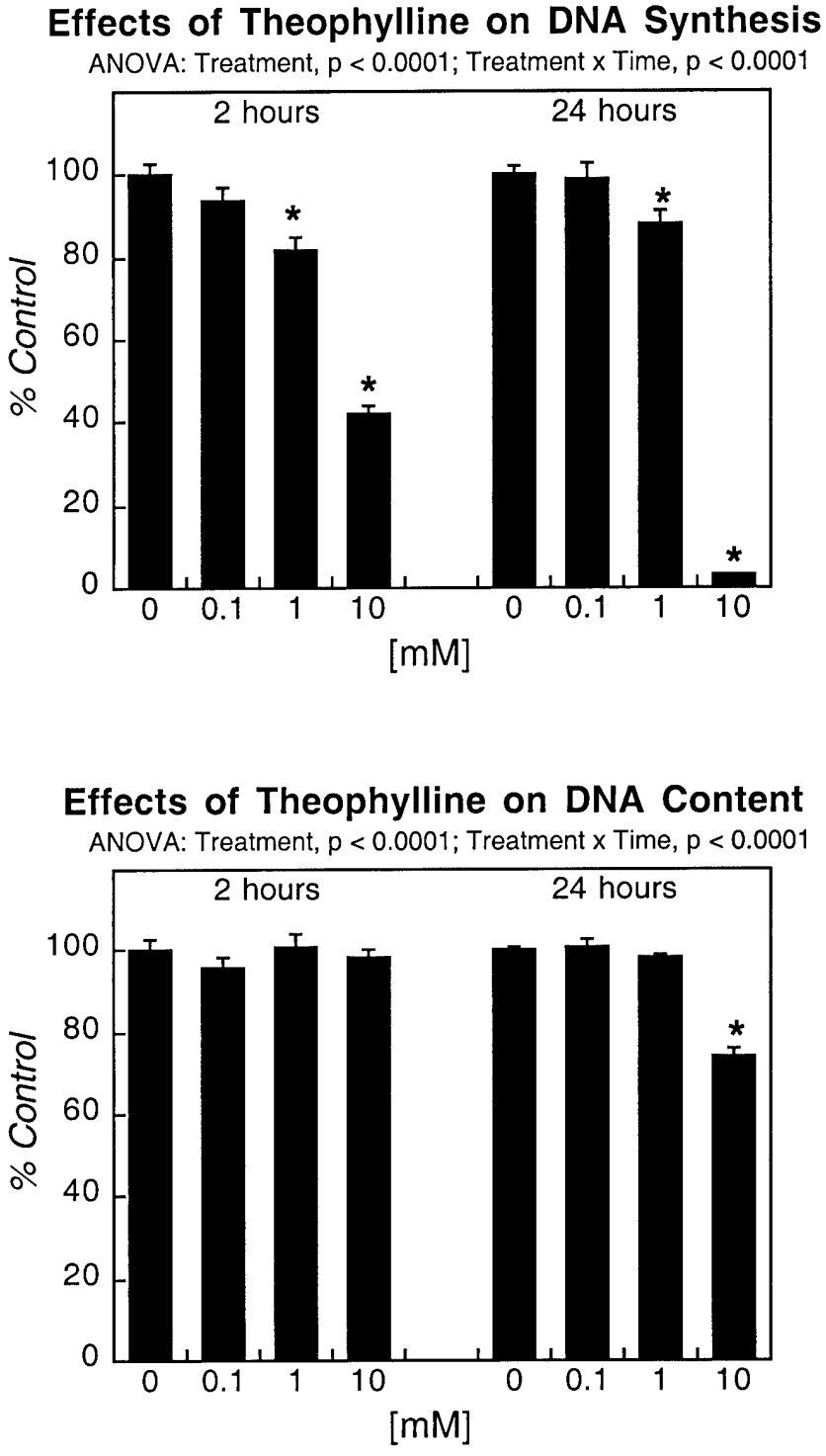

Effects of Theophylline on DNA Synthesis
ANOVA: Treatment, p < 0.0001; Treatment x Time, p < 0.0001
2 hours
24 hours
% Control
100
80
60
40
20
0
*
0
0.1
1
10
[mM]
Effects of Theophylline on DNA Content
ANOVA: Treatment, p < 0.0001; Treatment x Time, p < 0.0001
2 hours
24 hours
% Control
[mM]

Fig. 13

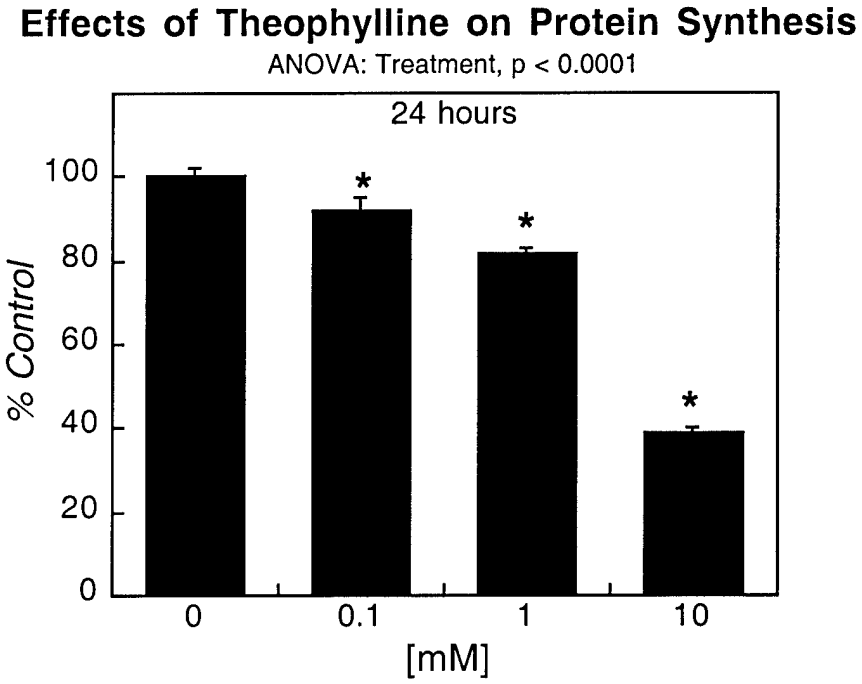

Effects of Theophylline on Protein Synthesis
ANOVA: Treatment, p < 0.0001
24 hours
% Control
100
80
60
40
20
0
*
*
*
0
0.1
1
10
[mM]

Fig. 14

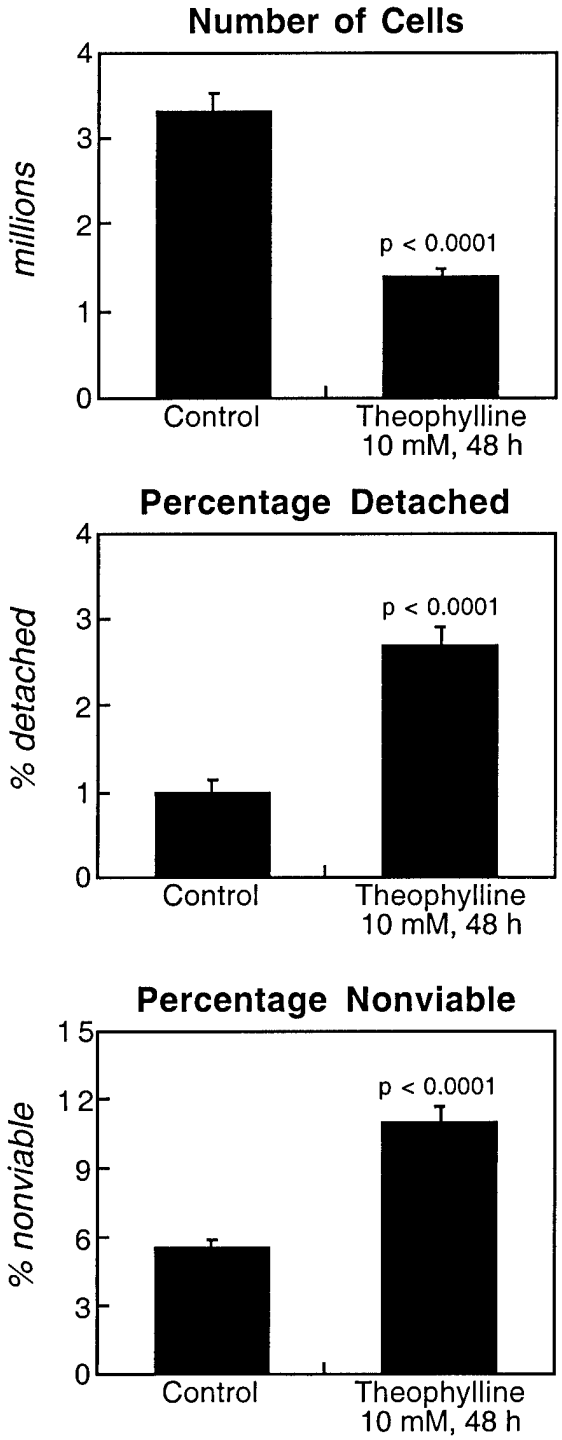

Number of Cells
millions
4
3
2
1
0
p < 0.0001
Control
Theophylline
10 mM, 48 h
Percentage Detached
% detached
4
3
2
1
0
p < 0.0001
Control
Theophylline
10 mM, 48 h
Percentage Nonviable
% nonviable
15
12
9
6
3
0
p < 0.0001
Control
Theophylline
10 mM, 48 h

Fig. 15

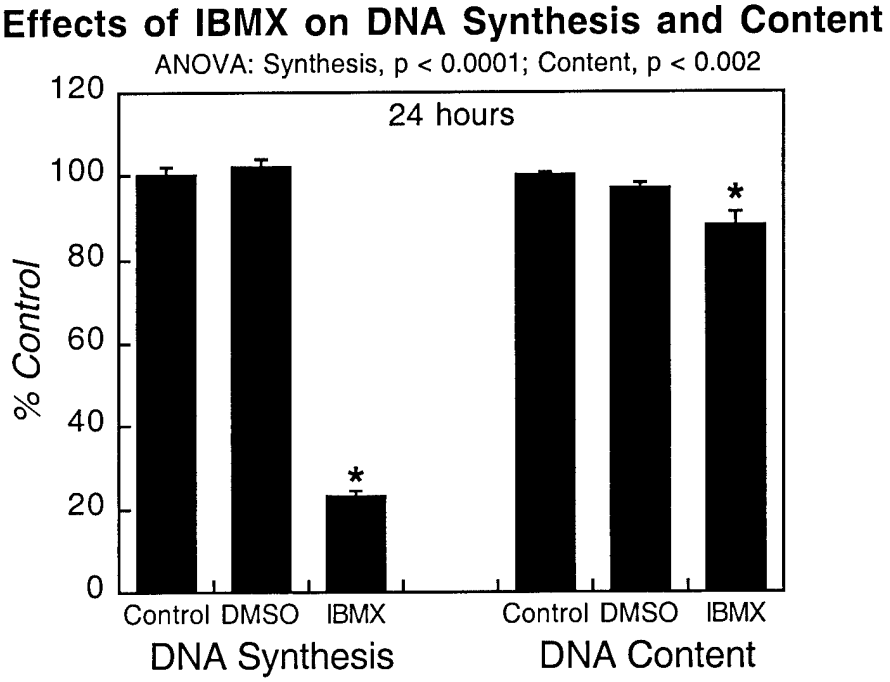

Effects of IBMX on DNA Synthesis and Content
ANOVA: Synthesis, p < 0.0001; Content, p < 0.002
24 hours
% Control
120
100
80
60
40
20
0
*
*
Control DMSO IBMX
Control DMSO IBMX
DNA Synthesis
DNA Content

Fig. 16

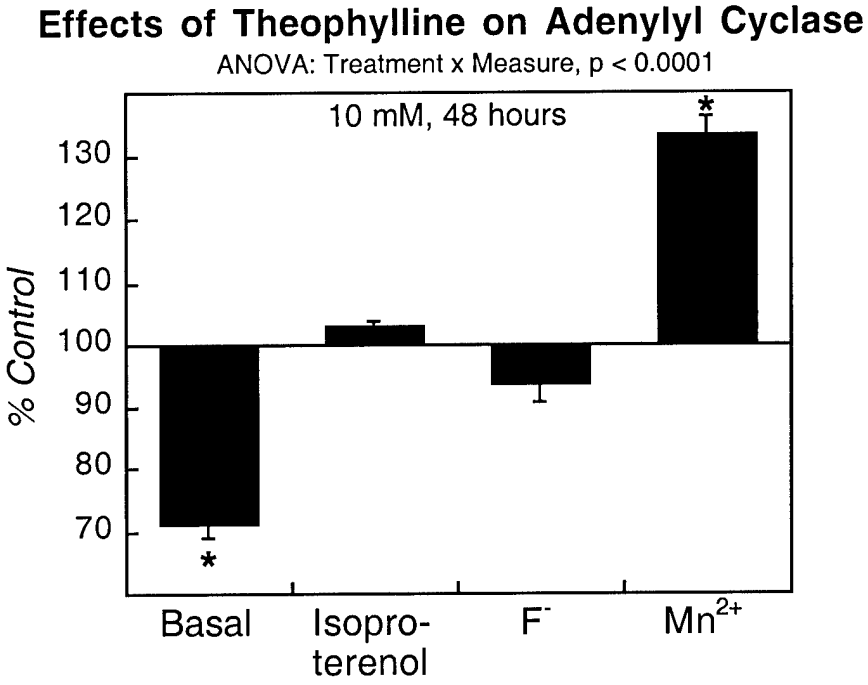
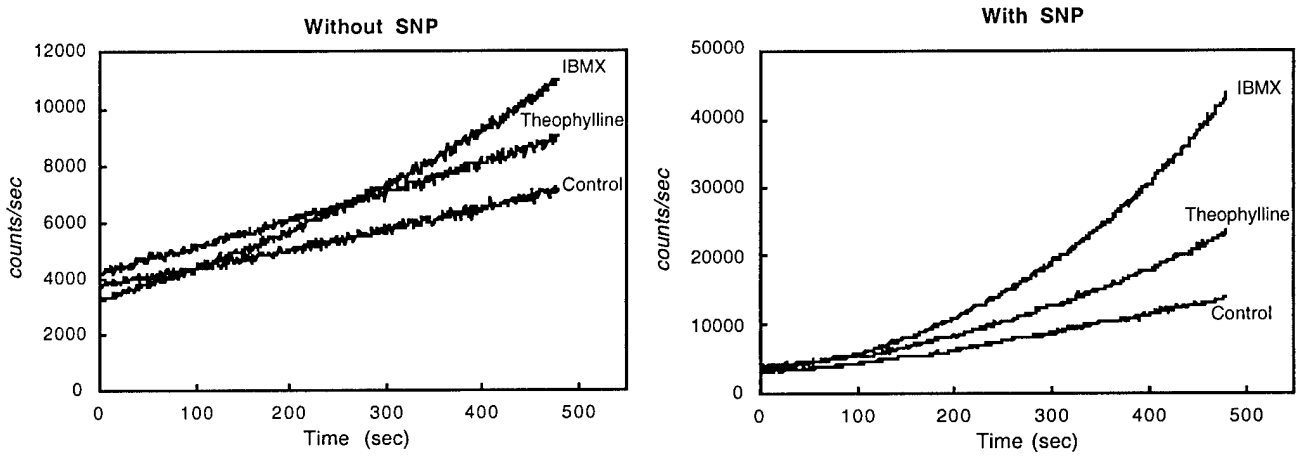
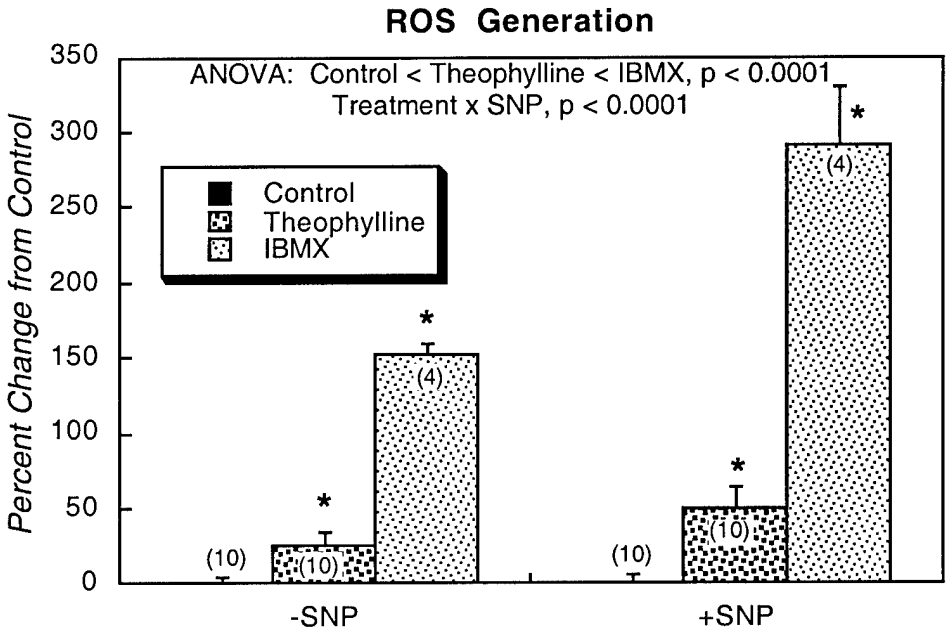

Effects of Theophylline on Adenylyl Cyclase
ANOVA: Treatment x Measure, p < 0.0001
10 mM, 48 hours
% Control
130
120
110
100
90
80
70
*
*
Basal
Isopro-terenol
F-
Mn2+

Fig. 17


Without SNP
With SNP
IBMX
Theophylline
Control
counts/sec
Time (sec)
0
2000
4000
6000
8000
10000
12000
20000
30000
40000
50000
100
200
300
400
500


ROS Generation
ANOVA: Control < Theophylline < IBMX, p < 0.0001
Treatment x SNP, p < 0.0001
Control
Theophylline
IBMX
Percent Change from Control
0
50
100
150
200
250
300
350
(10)
(4)
*
-SNP
+SNP

KEY RESEARCH ACCOMPLISHMENTS

- β-Adrenoceptors (βAR) are present on the cell surface in a number of different human breast cancer cell lines, including those that are nonresponsive to estrogen, and that are thus resistant to antiestrogen therapy.

- Exposure of MDA MB-231 human breast cancer cells to isoproterenol, a βAR agonist, caused an immediate reduction in DNA synthesis, an effect mediated through the generation of cyclic AMP within the cell.. The effect was mimicked by administration of membrane permeable cyclic AMP analogs, and showed additive actions with glucocorticoids. Unlike effects in normal cells, the response to β-agonists did not desensitize.

- Exposure of the cells to theophylline, which causes an even greater accumulation of cyclic AMP (through inhibition of phosphodiesterase, the enzyme that breaks down cyclic AMP), evoked total mitotic arrest and a profound reduction in cell number

- Theophylline not only arrested cell division, but also evoked cell death through a combination of cytotoxicity and oxidative stress.

- Because theophylline did not evoke desensitization of adenylyl cyclase, this drug can be used in conjunction with β-agonists to arrest the division of MDA-MB-231 breast cancer cells and to evoke cell death.

- Therapies targeting βAR and cyclic AMP may offer a chance to slow the growth and spread of some estrogen nonresponsive breast cancers. Since the effects on the cancer cells do not desensitize, whereas effects on normal cells do, effective regimens may be designed that minimize side effects. Attacking a cell surface receptor and its intracellular signaling molecules should remain an effective approach even after the emergence of multidrug resistance, which reduces the effectiveness only of drugs targeting intracellular sites.

REPORTABLE OUTCOMES

Publications:

Slotkin, T.A., Zhang, J., Dancel, R., Garcia, S.J., Willis, C. and Seidler, F.J., β-Adrenoceptor signaling and its control of cell replication in MDA-MB-231 human breast cancer cells, *Breast Canc. Res. Treat.* 60 (2000) 153-166.

Slotkin, T.A. and Seidler, F.J., Antimitotic and cytotoxic effects of theophylline in MDA-MB-231 human breast cancer cells, *Breast Canc. Res. Treat.* 64 (2000) 259-267.

Crumpton, T.L., Seidler, F.J. and Slotkin, T.A., Generation of reactive oxygen species by xanthine derivatives in MDA-MB-231 human breast cancer cells, *Breast Canc. Res. Treat.* 66 (2001) 143-146.

Slotkin, T.A., J. Zhang, R. Dancel, S.J. Garcia, C. Willis and F.J. Seidler, β-Adrenoceptor signaling and its control of cell replication in MDA-MB-231 human breast cancer cells. *Era of Hope Proceedings* **1**: 403, 2000.

Grant Proposals:
(Pending application) Beta-Adrenoceptor/Adenylyl Cyclase Signaling Cascade as a Target for Novel Chemotherapeutic Interventions in Breast Cancer. T.A. Slotkin (PI, 10% effort). US Army Medical Research, 7/1/02 - 6/30/015. Control of cell replication by neurotransmitter receptors expressed by human breast cancer cells. A number of human breast cancers manufacture β-adrenergic receptors. These receptors respond to stimulation by the "stress" hormones, norepinephrine and epinephrine, by ceasing division. We propose to determine how widespread the receptors are on different breast cancer cell lines and to examine the therapeutic responses to drugs targeting the receptors or their signaling target, adenylyl cyclase.

CONCLUSIONS

Year 1

Among the epithelial, endocrine and secretory cancer cell lines that express β-adrenoceptors, MDA-MB-231 human breast cancer cells exhibit comparatively high concentrations [15,31,36,37,55]. The B_{max} found here, 350 fmol/mg protein, exceeds the receptor concentration found in typical noradrenergic target tissues by over an order of magnitude [52] and is similar to the level found in brain regions enriched in noradrenergic projections. It is thus of critical interest that stimulation of these receptors leads to immediate inhibition of DNA synthesis and, with prolonged exposure, reductions in the total number of cancer cells; inhibition of DNA synthesis is a reliable predictor of chemotherapeutic responses in breast cancer cells [1]. β-Adrenoceptor-mediated inhibition of DNA synthesis was not shared by another cancer cell line, C6 rat glioma, that expresses a different β-receptor subtype at lower levels: whereas we found that MDA-MB-231 cells express β_2-receptors exclusively, C6 cells express primarily the β_1-subtype [22], but it is not clear whether this is the sole determinant of the role of β-adrenoceptors in the control of mitosis. Although there has been no systematic screening of breast cancer cell lines for β-adrenoceptor expression, these receptors have been identified in both estrogen-dependent and estrogen-independent types, including CG-5, BT-20, T47-D, VHB-1 and MCF-7 [36,37,55], as well as in tumors induced by administration by dimethylbenz(*a*)anthracene [31] and in unspecified collections of human breast cancer tissue samples [15]. In the two cases where receptor subtypes have been identified, the β_2-subtype appears to predominate, just as found here [15,37], although unlike our findings with MDA-MB-231 cells, some cell lines also make significant amounts of β_1-receptors [37]. In the CG-5 cell line, which expresses both the receptor subtypes, but at lower concentrations than those found here for MDA-MB-231 cells, administration of a β-agonist evokes a biphasic response, with enhanced cell replication at low concentrations and inhibition at high concentrations. Nevertheless, it is generally the case that increased cAMP, whether induced by receptor activation or by addition of membrane-permeable

cAMP analogs, inhibits cell replication and evokes tumor regression [15,36] and that β-receptor expression is predictive of the overall pharmacologic response [31]. Accordingly, an understanding of the specific role of β-adrenoceptor function in different breast cancer cell lines will require evaluation of receptor concentration, subtype, the efficiency of coupling to the generation of cAMP, and, as evaluated here, the persistence of receptor activity in the face of agonist-induced receptor downregulation and desensitization.

Isoproterenol-induced inhibition of DNA synthesis in the MDA-MB-231 breast cancer cell line exhibited the characteristic properties of β-receptor actions mediated through adenylyl cyclase: complete blockade by propranolol, and sharing of the effect by a membrane-permeable cAMP analog. What was unexpected, however, was the fact that isoproterenol's effect did not disappear with prolonged treatment, and in fact, was maintained at exactly the same level as the initial effect. Ordinarily, β-adrenoceptor agonists elicit downregulation and desensitization over a time frame of a few minutes to hours [50], and therefore a loss of effect was expected here. The first hint of atypical regulation of β-receptor signaling was provided simply by monitoring adenylyl cyclase responses under control conditions. Over a course of several days in culture, adenylyl cyclase activity declined, accompanied by a loss of responsiveness to stimulants acting either at the level of β-receptors (isoproterenol), G-proteins (fluoride), or cyclase itself (Mn^{2+}). However, the enzymatic response to isoproterenol showed a smaller decline than for any other stimulant, that is, the β-adrenoceptor response actually increased relative to the total amount of catalytic activity. In fact, after 3-4 days in culture, isoproterenol elicited the maximal possible activation of adenylyl cyclase, since the activity was indistinguishable from that seen with addition of Mn^{2+}. In addition, the response to isoproterenol, which selectively activates G_s, was significantly greater than that to fluoride, which causes activation of both stimulatory and inhibitory G-proteins. The profound response to isoproterenol is unusual, since in most tissues, isoproterenol is incapable of eliciting adenylyl cyclase activation equaling that of direct G-protein or cyclase stimulants [19,35,59,66]. The number of β-receptors also kept pace with cell division and growth, so that the overall concentration of receptors remained unchanged throughout 4 days in culture, a period in which the number and size of cells increased substantially.

Given the maintenance of the ability of isoproterenol to inhibit DNA synthesis, we expected to see failure of receptor downregulation and/or desensitization, paralleling the situation during differentiation of normal cells possessing these receptors [19,35,66]. Surprisingly, isoproterenol caused immediate and robust receptor downregulation, accompanied by a parallel loss of the ability of receptor activation to stimulate adenylyl cyclase activity. Downregulation reached over 90% within 24h and the adenylyl cyclase response was desensitized by 75% throughout the period in which inhibition of DNA synthesis was maintained. These results thus indicate that downregulation and desensitization do occur, but that the ability of receptor stimulation to inhibit DNA synthesis and cell acquisition requires activation of only a very small number of receptors. This raises the possibility that there may be adaptations in the signaling pathway downstream from receptors, G-proteins and cyclase, that serve to maintain the net effect of receptor stimulation. In keeping with this view, we have found that, during brain development, the initial stimulation of β-receptors "programs" cAMP response elements so as to preserve or

enhance the response of gene expression to adrenergic input [59,60]. If similar events occur in MDA-MB-231 cells, then adaptations of downstream elements may preserve the effects on cell replication in the face of receptor downregulation and desensitization. Accordingly, a logical next step is to look at transcription factors and genes targeted by β-receptor stimulation and their role in the antimitotic effect of isoproterenol.

From both the standpoints of mechanism and therapeutics, our findings of augmented effects with cotreatment of dexamethasone or theophylline are potentially important. Initially, we expected dexamethasone to enhance the response to isoproterenol because glucocorticoids induce the synthesis of β_2-receptors in normal cells [14], especially during cell differentiation [43]. Dexamethasone treatment by itself inhibited DNA synthesis in MDA-MB-231 cells leading eventually to a reduction in cell number. In addition, when dexamethasone was combined with isoproterenol treatment, we saw even greater inhibition of DNA synthesis and loss of cells. However, when we examined the mechanism underlying the combined effect, we found that dexamethasone was not capable of preventing agonist-induced β-receptor downregulation or desensitization; in fact, the combination of dexamethasone and isoproterenol produced a more-than-additive receptor downregulation. The disparity between the augmented effects of combined dexamethasone and isoproterenol treatment on DNA synthesis, which were less than additive, and effects directed toward receptor expression, which were synergistic and in a direction opposite to that expected from the effects on cell replication, indicate that dexamethasone must be acting on elements downstream from the receptor. Indeed, earlier work in replicating and differentiating cells indicates that glucocorticoids induce G-proteins and adenylyl cyclase [43]. In the current study, we found significant increases in total adenylyl cyclase activity (*i.e.* enhanced response to Mn^{2+}) after combined treatment with isoproterenol and dexamethasone; although dexamethasone and isoproterenol individually produced receptor uncoupling from adenylyl cyclase, dexamethasone did not augment the uncoupling caused by isoproterenol, even in the face of greater receptor downregulation. Obviously, post-receptor targets, including but not limited to adenylyl cyclase, play a pivotal role in maintaining the net response to receptor stimulation in the face of extensive downregulation. Additional downstream factors, such as protein kinase A and phosphorylated target proteins, are necessary to couple β-adrenoceptors to effects on cell replication and differentiation, and future studies will need to determine how glucocorticoids alter their expression and/or function to maintain the antimitotic response to receptor stimulation.

The second cotreatment that we examined was the phosphodiesterase inhibitor, theophylline. This drug alone had the greatest effect on mitosis: after several days in culture, the number of cells appeared to be no greater than that originally plated. Theophylline has a greater effect than isoproterenol because it interrupts the ability of phosphodiesterase to limit the rise of intracellular cAMP levels. Indeed, the effects of theophylline are precisely the same as those seen for effects of prolonged elevations of cAMP during normal cell development, namely termination of cell division in favor of cell growth and differentiation [11,17,41], the same type of effects seen for cAMP in several other cancer cell lines [9,33]. Accordingly, the theophylline-treated cells also showed gross enlargement as well as a morphological change to a stellate

appearance, confirming the separability of effects on mitosis from those on cell growth and differentiation. In light of the findings for isoproterenol and dexamethasone, it is again possible that theophylline may influence gene expression downstream from cAMP generation, or alternatively, may act through other surface receptors (*e.g.* adenosine receptors) whose expression has not been explored in these cell lines.

Regardless of the ancillary mechanisms involved in β-adrenoceptor-mediated inhibition of mitosis in MDA-MB-231 breast cancer cells, the fact that inhibition does not disappear with receptor downregulation and desensitization raises the possibility for therapeutic strategies employing receptor agonists, alone or in combination with glucocorticoids and phosphodiesterase inhibitors. The cell line studied here, for example, is estrogen-insensitive and is thus nonresponsive to standard antiestrogen therapies. Furthermore, interventions operating at the level of cell surface receptors, such as β-adrenoceptors, do not require penetration of drug to the interior of the cell, and thus would not be subject to loss of effect from induction of transporters in multidrug resistance. Although theophylline was more effective than isoproterenol in reducing the number of cells in culture, it is distinctly possible that it would prove less effective with prolonged treatment *in vivo*. If theophylline acts through inhibition of phosphodiesterase, an intracellular locus, the cells may develop resistance, whereas if its actions are directed toward cell surface receptors (*e.g.* adenosine receptors), theophylline should maintain its effectiveness. From a mechanistic standpoint, input from receptors on the cell surface, like β-adrenoceptors, is "upstream" from genes whose mutations lead to constitutive activation and cell proliferation [32,34,60]; receptor stimulation could thus limit the net effects of adverse mutations on cell cycle control by restricting expression of these genes. Given that desensitization effectively terminates the physiological effects of β-agonists in normal cells, therapeutic interventions based on receptor targeting should have only short-term side effects relative to the maintenance of effect in the target cell population. The concentrations of isoproterenol found to elicit inhibition of DNA synthesis and reductions of cell number in the current study (nM - μM) lie well within the range compatible with β-adrenergic effects in a wide variety of *in vivo* and *in vitro* systems [2] and approximates K_d values of isoproterenol for typical β-adrenoceptors [4,12]. Consequently, it may be feasible to produce effective β-agonist concentrations for *in vivo* application; furthermore, the pharmacokinetics and toxicity of these agents are well-established. Neurotransmitter-based therapeutic strategies should thus be explored in an *in vivo* model to establish the potential utility of this general approach. Indeed, similar work with neuropeptides targeting opioid receptors has established their ability to control cell growth and replication in colon cancer [23,64]. In the current case, screening of human cancers for the presence of β-adrenoceptors, or other cAMP-linked neurotransmitter receptors, along with evaluation of the response of the cells to receptor agonists or antagonists, may establish new treatment strategies.

Year 2

In our previous work with MDA-MB-231 cells, we found that increasing the intracellular cAMP concentration led to a decrease in mitosis, so that membrane-permeable cAMP analogs, a β-adrenoceptor agonist (isoproterenol), or a phosphodiesterase inhibitor (theophylline), all led to a

decrease in cell number [48]. However, theophylline was far more effective than any other treatment and moreover, induced major morphologic changes not seen with the other agents. The current results indicate that theophylline has collateral actions that affect cell number and differentiate state. A comparison of the results of inhibition of DNA synthesis by theophylline, and the eventual reduction in cell number, provides the first indication of these additional effects. Based on the observed doubling time of MDA-MB-231 cells under our culture conditions, complete mitotic arrest for a 48h span would result in a 35-40% deficit in total cell number. However, 10 mM theophylline caused a significantly greater reduction: at 48h, the theophylline had only 40% of the control cell number; by 72h, measurements of DNA content indicated a deficit of over 80%, whereas mitotic arrest predicts only a 50% deficit. The unexpectedly large deficit in cell number is even more impressive in light of the fact that theophylline did not cause complete mitotic arrest, but rather elicited only 60% inhibition in the initial exposure period. Indeed, the fact that theophylline's effects on DNA synthesis intensify over a 24h span, implies that subsequent cellular changes are occurring that impact on cell division.

These results suggest that theophylline impacts events over and above the direct effect on cAMP and hence on cell replication. Our findings for protein synthesis and viability confirm this conclusion. Protein synthesis was reduced after a 24h exposure to theophylline at concentrations that did not affect DNA synthesis; furthermore, the entire concentration-response curve for effects on protein synthesis differed from that on DNA synthesis, as the maximal effect toward protein was considerably less. The studies with cell attachment and trypan blue exclusion provide further evidence that theophylline has an effect on cell viability in addition to its effects on replication. Theophylline exposure more than doubled the proportions of detached cells and of nonviable cells. It is unlikely that increased cAMP levels consequent to phosphodiesterase inhibition, can solely account for these findings; when we compared the effects of theophylline with those of IBMX, another xanthine-based, phosphodiesterase inhibitor, we obtained results indicative of actions separable from those on cAMP. A concentration (1 mM) of IBMX that produced inhibition of DNA synthesis equivalent to that seen with 1-10 mM theophylline failed to evoke a decrease in cell number beyond what would have been expected from mitotic inhibition, a situation very different from the effects of theophylline.

There are two likely supplementary mechanisms for theophylline's actions on cell viability: formation of oxidative free radicals, and actions at adenosine receptors. Theophylline is a substrate for xanthine oxidase, an enzyme known to generate free radicals and resultant cell damage [30,51,58,63]. However, IBMX is also a xanthine and similarly induces free radical formation [58]. Thus, either theophylline is more potent than IBMX in eliciting oxidative stress, or alternatively, it elicits cytotoxicity through collateral mechanisms, such as actions on adenosine receptors [5]. Indeed, free radicals produced by xanthine oxidase tend to increase cancer cell adhesion [51], whereas a decrease was seen with theophylline in MDA-MB-231 cells, implying that free radicals alone cannot account for all aspects of theophylline's cytotoxicity. The alternative, namely a role of adenosine receptors in cancer cell replication, differentiation and cytotoxicity has been explored only sporadically. Activation of the receptors reduces cell replication [25] and theophylline, acting as an antagonist, might then be expected to enhance, not

inhibit mitosis. However, recent work indicates that under varying conditions of cell differentiation, theophylline derivatives possess mixed agonist-antagonist properties that can contribute to cytotoxicity [5]. It is thus likely that the effects of theophylline on MDA-MB-231 cells represents the summation of three different mechanisms: phosphodiesterase inhibition, free radical formation, and actions at adenosine receptors.

All three mechanisms are also likely to contribute to the distinct changes in cell morphology and function seen after theophylline exposure [48]. Oxidative stress itself induces cell differentiation [24]; since the increase in cAMP evoked by phosphodiesterase inhibition also serves to switch cells from replication to differentiation [9,11,17,33,41,48], the combined effect can account for the profound change in morphology evoked by theophylline but not by treatments that have more modest effects restricted only to cAMP [48]. The morphological changes elicited by theophylline suggest a profound change in differentiation state, and we obtained evidence for altered cell reactivity after theophylline exposure, another characteristic of differentiation. The adenylyl cyclase signaling cascade undergoes substantial changes during growth of MDA-MB-231 cells, characterized by a loss of basal enzyme activity but preservation of signaling mediated through membrane-bound β-adrenoceptors [48]. Treatment of the cells with theophylline similarly evoked a decrease in basal enzyme activity with maintenance of the response to stimulation of β-adrenoceptors by isoproterenol, or to fluoride-induced stimulation of the G-proteins that couple the receptors to adenylyl cyclase. Furthermore, there was specific enhancement of the response to manganese, which operates directly on adenylyl cyclase itself, without the participation of receptors or G-proteins; this implies either that adenylyl cyclase is induced by theophylline, or alternatively, that there is a shift toward expression of a more active isoform. In either case, the promotional effect on adenylyl cyclase is likely to augment the effects of theophylline on cAMP generation, augmenting the effect of phosphodiesterase inhibition. The induction of adenylyl cyclase and preservation of the β-adrenoceptor effect throughout differentiation may contribute additionally to the maintained ability of isoproterenol to inhibit MDA-MB-231 cell replication in the face of receptor downregulation that would ordinarily limit the response, thus augmenting the antitumor effect of other potential therapeutic interventions [48].

The multiple pharmacologic properties of theophylline, producing mitotic inhibition, cytotoxicity and altered signaling in MDA-MB-231 cells, may provide insight into novel therapeutic strategies. In light of the antitumor effects seen here, it may be worthwhile to reexamine the potential chemotherapeutic use of xanthine derivatives.

Year 3

Our studies with ROS formation indicate there are two distinct effects of the xanthine derivatives contributing to oxidative stress in MDA-MB-231 cells. Both theophylline and IBMX evoke ROS formation by themselves, and in addition, they enhance the oxidative response to SNP, indicating that the xanthines sensitize the cells to other oxidative stressors. These results suggest that xanthine derivatives should be designed to emphasize oxidative responses; given the higher

metabolic rate of cancer cells, tumor cells are likely to be especially targeted for such actions, given that derivatives can be obtained which will enhance tumor selectivity and minimize normal tissue toxicities. Because the xanthines sensitize the cells to other oxidative stressors, there is also an opportunity to design combination therapies targeting ROS formation.

Although IBMX is a far more potent phosphodiesterase inhibitor than theophylline [54] and also proved to be much more effective in generating ROS, we found previously that theophylline was at least as effective as IBMX in inhibiting growth of MDA-MB-231 cells [45], suggesting that a third class of xanthine effects may represent an additional therapeutic target. One possibility is the activation of adenosine to elicit apoptosis [42]. In normal cells, this occurs through the A3 subtype, for which neither theophylline nor IBMX are particularly effective. Accordingly, if apoptosis occurs through adenosine receptor-mediated mechanisms in MDA-MB-231 cells, it would require either the presence of atypical receptors, or alternatively, activation of other receptor subtypes could be linked to apoptosis in these cells. In comparing phosphodiesterase inhibition, ROS formation and adenosine receptor actions, it is also important to note the differences in concentrations required for each effect. The K_d for theophylline's actions on adenosine receptors other than the A3 subtype lies in the μM range [10], whereas its K_i for inhibition of phosphodiesterase requires mM concentrations [45,54]. Our current results indicate that ROS formation occurs at concentrations intermediate to the two other potential mechanisms. In clinical settings, theophylline exhibits significant side effects at concentrations above 100 μM, obviously below the concentration necessary for phosphodiesterase inhibition. However, cytotoxicity and inhibition of cell replication occur at the lower concentrations commensurate with adenosine receptor actions and possibly, as seen here, ROS generation [45,48]. Therefore, in the discovery process for new antitumor agents it might be useful to pursue the design of xanthine derivatives emphasizing the latter two mechanisms as opposed to phosphodiesterase inhibition. In that regard, compounds resembling IBMX are likely to be much more effective than theophylline.

"SO WHAT"

Regardless of the ancillary mechanisms involved in β-adrenoceptor-mediated inhibition of mitosis or in the cytotoxic and antimitotic effects of theophylline in MDA-MB-231 breast cancer cells, the fact that these drugs do arrest mitosis and lead to cell death, without loss of response despite receptor downregulation and desensitization, raises the possibility for therapeutic strategies employing these agents. The cell line studied here, for example, is estrogen-insensitive and is thus nonresponsive to standard antiestrogen therapies. Furthermore, interventions operating at the level of cell surface receptors, such as β-adrenoceptors, do not require penetration of drug to the interior of the cell, and thus would not be subject to loss of effect from induction of transporters in multidrug resistance. From a mechanistic standpoint, receptor input and the associated cell signaling cascade (in this case, cyclic AMP) is "upstream" from genes, whose mutations lead to constitutive activation and cell proliferation; cyclic AMP generation could thus limit the net effects of adverse mutations on cell cycle control by restricting expression of these genes. Given that desensitization effectively terminates the physiological effects of β-agonists in normal cells, therapeutic interventions based on receptor targeting should have only short-term

side effects relative to the maintenance of effect in the target cell population; furthermore, the pharmacokinetics and toxicity of β-agonists and theophylline are well-established, since these drugs have long been used to treat asthma or to arrest premature labor. Neurotransmitter and cell signaling-based therapeutic strategies should be explored in an *in vivo* model to establish the potential utility of this general approach. If successful, screening of human cancers for the presence of β-adrenoceptors, or indeed, of other cell surface receptors linked to cyclic AMP, along with *in vitro* evaluation of the response of the cells to receptor agonists or antagonists, may establish new treatment strategies.

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PERSONNEL RECEIVING PAY FROM THIS PROJECT

Theodore Slotkin (PI)
Jian Zhang (technical staff)
Dan Qiao (technical staff)
Mandy Cousins (technical staff)
Channelle Christopher (technical staff)

APPENDICES

Slotkin, T.A., Zhang, J., Dancel, R., Garcia, S.J., Willis, C. and Seidler, F.J., β-Adrenoceptor signaling and its control of cell replication in MDA-MB-231 human breast cancer cells, *Breast Canc. Res. Treat.* 60 (2000) 153-166.

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Report

β-adrenoceptor signaling and its control of cell replication in MDA-MB-231 human breast cancer cells

Theodore A. Slotkin, Jian Zhang, Ria Dancel, Stephanie J. Garcia, Carrie Willis, and Frederic J. Seidler

Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC, USA

Key words: adenylyl cyclase, β-adrenoceptors, cell replication, cyclic AMP, DNA synthesis, glucocorticoids

Summary

MDA-MB-231 human breast cancer cells express high β-adrenoceptor levels, predominantly the β_2 subtype. Receptor stimulation by isoproterenol evoked immediate reductions in DNA synthesis which were blocked completely by propranolol and were of the same magnitude as effects elicited by high concentrations of 8-Br-cAMP. Isoproterenol-induced inhibition of DNA synthesis was maintained throughout several days of exposure, resulting in a decrement in total cell number, and the effects were augmented by cotreatment with dexamethasone; an even greater effect was seen when cAMP breakdown was inhibited by theophylline, with or without addition of isoproterenol. Despite the persistent effect of isoproterenol, receptor downregulation was evident with as little as 1 h of treatment, and over 90% of the receptors were lost within 24 h. Receptor downregulation was paralleled by homologous desensitization of the adenylyl cyclase response to β-adrenoceptor stimulation. Dexamethasone augmented the effects of isoproterenol on DNA synthesis but did not prevent receptor downregulation or desensitization. These results indicate that β-adrenoceptors are effectively linked, through cAMP, to the termination of cell replication in MDA-MB-231 human breast cancer cells, and that activation of only a small number of receptors is sufficient for a maximal effect. Novel pharmacologic strategies that focus on cell surface receptors operating through adenylyl cyclase may offer opportunities to combat cancers that are unresponsive to hormonal agents, or that have developed multidrug resistance.

Abbreviations: ANCOVA: analysis of covariance; ANOVA: analysis of variance; cAMP: cyclic adenosine-3′,5′-monophosphate

Introduction

In addition to their role as neurotransmitters and 'stress' hormones, catecholamines play a trophic role in the control of cell replication and differentiation in target cells that express adrenergic receptors. Lower organisms, such as sea urchins, overexpress norepinephrine, epinephrine, and other biogenic amines during critical developmental periods in which these amines control cell replication and differentiation [1]. In mammals, 'spikes' of adrenergic activity also modulate the rate of cell replication and differentiation and thus control the architectural modeling of adrenergic target tissues [2–6]. The importance of adrenergic control of mammalian cell development has recently been pointed out by the lethal effects of gene knockouts that eliminate the ability to synthesize norepinephrine or to express β-receptors; these animals die *in utero* from disruption of cardiac cell replication/differentiation and consequent dysmorphogenesis [7–9]. The critical period for adrenergic control of these events terminates as cells exit mitosis and approach terminal differentiation, so that the sensitivity to adrenergic effects on cell replication disappears in adulthood except for a few tissues that undergo continual renewal [4, 5, 10, 11].

It is, thus, of critical importance that, with carcinogenic redifferentiation, many cell types, including

epithelial cancers and cancers of secretory cells, re-express β-adrenergic receptors [12–16], which can once again resume their role in the control of cell replication [13, 16, 17]. In some cell lines, β-adrenergic stimulation elicits a small, promotional effect on cell replication [13, 17, 18], whereas in others, stimulation of these receptors and the consequent rise in intracellular cAMP levels inhibits mitosis [16, 19]. β-adrenoceptors on cancer cells, thus, recapitulate both the promotional and inhibitory roles of these receptors in cell replication seen in the development of normal cells [4–6, 20, 21]. Accordingly, it might be feasible to use β-adrenoceptor agonists or antagonists as pharmacologic interventions to control the replication of cancer cells. Indeed, short-term isoproterenol treatment of PC-3 prostate cancer cells inhibits DNA synthesis through β-receptor-mediated increases in cAMP [16], and direct administration of membrane permeable cAMP analogs inhibits tumorigenesis of MCF-7 breast cancer cells [19].

Receptor downregulation and desensitization are major problems limiting the potential use of β-receptor agonists to control cell replication. Ordinarily, prolonged receptor stimulation uncouples receptors from response elements (desensitization) and leads to internalization and sequestration of receptor proteins (downregulation), limiting the intensity and duration of cell stimulation [22]. During normal development, however, we have found that these processes are poorly developed so that responses are maintained or enhanced with agonist treatment [23–25]. This raises the possibility that loss of response may not occur in cancer cells as well. In the current study, we evaluate that hypothesis using MDA-MB-231 cells, a human breast cancer line that expresses high levels of β-adrenoceptors [26]. We report that prolonged β-agonist administration maintains inhibition of DNA synthesis and suppresses cell replication even when only a small proportion of the receptors remains, so that desensitization and downregulation do not limit the effect. We also show that effects are augmented by glucocorticoids, just as is true for normal cells during development [27], and also by inhibition of cAMP breakdown by theophylline.

Methods

MDA-MB-231 cells (Duke University Comprehensive Cancer Center, Durham, NC) were seeded at a density of 10^6 cells per 100 mm diameter dish and maintained in modified minimum essential medium containing Earle's salts, 5% fetal bovine serum, 2 mM glutamine, 100 IU/ml of penicillin, 0.1 mg/ml of streptomycin and 5 μg/ml of insulin (all from Gibco, Grand Island, NY). Cells were incubated with 7.5% CO_2 at 37°C, and the medium was changed every 24 h. Cells were examined at 100× magnification for counting and morphological features. Each experiment was repeated several times with separate batches of cells, after an average of five passages. Each passage and preparation was verified for morphology, growth rate, and the expression and/or function of β-adrenoceptors. Treatment effects were always compared against concurrent control cultures from the same passage and batch of cells. Except as otherwise indicated, all drugs were obtained from Sigma Chemical Co. (St. Louis, MO).

DNA synthesis and content

To initiate the measurement of DNA synthesis, the medium was changed to include 1 μCi/ml of [^{3}H]thymidine (specific activity, 2 Ci/mmol; New England Nuclear, Boston, MA). Incubations were carried out for 1 h in the presence or absence of the appropriate drugs. At the end of that period, the medium was aspirated and cells were harvested in 3.5 ml of ice-cold water. Duplicate aliquots of each sample were treated with 10% trichloroacetic acid and sedimented at 1000 × *g* for 15 min to precipitate macromolecules and the resultant pellet was washed once with additional trichloroacetic acid and with 75% ethanol. The final pellet was then hydrolyzed with 1 M KOH overnight at 37°C, neutralized with HCl, and the DNA was then precipitated with ice-cold 5% trichloroacetic acid and sedimented at 1000 × *g* for 15 min. The pellet from this final step was hydrolyzed in 5% trichloroacetic acid for 15 min at 90°C, resedimented, and an aliquot of the supernatant solution counted for [^{3}H]thymidine incorporation. Another aliquot was assayed for DNA spectrophotometrically by absorbance at 260 nm. Previous work has demonstrated quantitative recovery of DNA by these techniques [28]. Incorporation values were corrected to the amount of DNA present in each culture to provide an index of DNA synthesis per cell.

β-adrenoceptor binding

The medium was removed and cells were washed once with ice-cold, calcium- and magnesium-free Earle's balanced salt solution. Fresh solution was added and

the cells were scraped off the dish and sedimented at 40,000 × *g* for 15 min. The pellet was resuspended (Polytron, Brinkmann Instruments, Westbury, NY) in 10 mM $MgCl_2$, and 50 mM Tris (pH 7.4) and the homogenate was sedimented at 40,000 × *g* for 15 min. The pellets were dispersed with a homogenizer (smooth glass fitted with a Teflon pestle) in the same buffer.

Each assay contained membrane suspension corresponding to ≈5 μg of protein and 67 pM [^{125}I]iodopindolol (specific activity 2200 Ci/mmol, New England Nuclear) in a final volume of 250 μl of 145 mM NaCl, 2 mM $MgCl_2$, 20 mM Tris (pH 7.5) and 1 mM ascorbate. Nonspecific binding was evaluated with identical samples containing 100 μM isoproterenol, and was typically 15% of the total binding. In some experiments, displacement of ligand binding was carried out with the specific β_1-receptor antagonist, CGP20712A (Research Biochemicals International, Natick, MA) to identify the receptor subtype present on MDA-MB-231 cells. Scatchard determinations to identify changes in receptor number (B_{max}) or affinity (K_d) were carried out over a range of [^{125}I]iodopindolol concentrations from 0.02 to 1 nM.

Adenylyl cyclase activity

Cell membranes were prepared by the same procedure as for β-receptor binding, except that the buffer consisted of 250 mM sucrose, 1 mM EGTA, 10 mM Tris (pH 7.4). Aliquots of membrane preparation containing ≈20 μg protein were then incubated for 30 min at 30°C with final concentrations of 100 mM Tris–HCl (pH 7.4), 10 mM theophylline, 1 mM adenosine 5′-triphosphate, 10 mM $MgCl_2$, 1 mg bovine serum albumin, and a creatine phosphokinase-ATP-regenerating system consisting of 10 mM sodium phosphocreatine and 8 IU phosphocreatine kinase, and 10 μM GTP in a total volume of 25 μl. The enzymatic reaction was stopped by placing the samples in a 90–100°C water bath for 5 min, followed by sedimentation at 3000 × *g* for 15 min, and the supernatant solution was assayed for cAMP using radioimmunoassay kits (Amersham Corp., Chicago, IL). Preliminary experiments showed that the enzymatic reaction was linear well beyond the assay time period and was linear with membrane protein concentration; concentrations of cofactors were optimal and, in particular, the addition of higher concentrations of GTP produced no further augmentation of activity. In addition to evaluating basal activity, the maximal total activity of the adenylyl cyclase catalytic unit was evaluated with the response to 10 mM $MnCl_2$ [29].

The contributions of G-protein-linked processes to adenylyl cyclase were evaluated in two ways. First, to determine the net G-protein-linked response of adenylyl cyclase activity with maximal activation of all G-proteins, samples were prepared containing 10 mM NaF in the presence of GTP [29]. Second, β-adrenoceptor-targeted effects mediated through the G-proteins were evaluated with 100 μM isoproterenol in the presence of GTP. The concentrations of all the agents used here have been found previously to be optimal for effects on adenylyl cyclase and were confirmed in preliminary experiments [29, 30].

Data analysis

Data are presented as means and standard errors. For each study, treatment-related differences were first evaluated by a global ANOVA, incorporating all variables in a single test. For studies of adenylyl cyclase activity, multiple measurements were made from the same membrane preparation since several different stimulants were compared; in that case, stimulant was considered a repeated measure. For studies of blockade of one drug by another, or of additive or synergistic effects, the combined effects were evaluated by two-factor ANOVA with the working hypothesis dependent upon a significant interaction between the two treatments. Where significant treatment effects were identified with the global test, individual differences between treatment groups were established with Fisher's protected least significant difference.

Scatchard plots were fitted by linear regression analysis and treatment-related differences were first compared by ANCOVA. Differences in maximal binding capacity (B_{max}) and the equilibrium dissociation constant (K_d, the reciprocal of receptor affinity) were then evaluated using Fisher's protected least significant difference.

Significance for main treatment effects was assumed at $p < 0.05$ and interaction terms were considered significant at $p < 0.1$ [31]. For convenience, some data are presented as a percentage of control values, but statistical significance was always assessed on the unmanipulated data. Where multiple time points are presented in the same graph, the control groups are given as a single value (100%), but statistical comparisons were conducted only with the time-matched group appropriate to each treatment.

Results

MDA-MB-231 cells were in log-phase growth from 1 to 4 days after plating, commencing at 25% confluence on day 1, through 80% confluence on day 4 (data not shown). Over this span, DNA synthesis was maintained at a nearly constant rate (7000 ± 500 dpm/μg DNA on day 1, 8100 ± 600 on day 4) and the number of cells, indicated by total DNA content, rose substantially (27 ± 2 μg DNA on day 1, 45 ± 3 μg on day 4). The increase in confluence between 1 and 4 days (more than double) was larger than the increase in DNA content (65%), indicating that cell enlargement was also occurring over this span. Drug treatments were initiated after one day in culture and were terminated at various times during log-phase growth, no later than four days in culture.

Addition of as little as 1 nM of isoproterenol to the medium produced immediate and robust inhibition of DNA synthesis (Figure 1). The effect was maximal at 100 nM isoproterenol and was maintained throughout a 48 h exposure. At the end of that period, isoproterenol-treated cells showed a significant reduction in the number of cells, assessed by DNA content. The antimitotic effect was not related to cytotoxicity or loss of viability. Trypan blue exclusion indicated 95.1 ± 0.5% viability after a 48 h to 10 μM isoproterenol, compared to 96.2 ± 0.5% in controls (NS, $n = 8$ for each treatment group); similarly, isoproterenol did not increase the proportion of detached cells (0.9 ± 0.1%, compared to 1.5 ± 0.3% in controls). To demonstrate that the effects of isoproterenol on DNA synthesis were mediated through β-adrenoceptors stimulating the production of cAMP, a comparison was made with the membrane permeable cAMP analog 8-Br-cAMP, and with the effects of the β-receptor antagonist propranolol (Figure 2). Isoproterenol and 8-Br-cAMP were equally effective toward DNA synthesis, and the effect of isoproterenol was completely blocked by propranolol. Propranolol by itself had no effect.

In developing tissues, glucocorticoid administration can sensitize cells to β-adrenoceptor agonists by inducing receptor formation and by enhancing signaling components of the adenylyl cyclase cascade [27]. Accordingly, we examined whether dexamethasone enhances the ability of isoproterenol to inhibit DNA synthesis and to reduce the number of cells (Figure 3). By itself, a 24 or 48 h pretreatment with dexamethasone caused 20% inhibition of DNA synthesis and a significant reduction in cell number. When

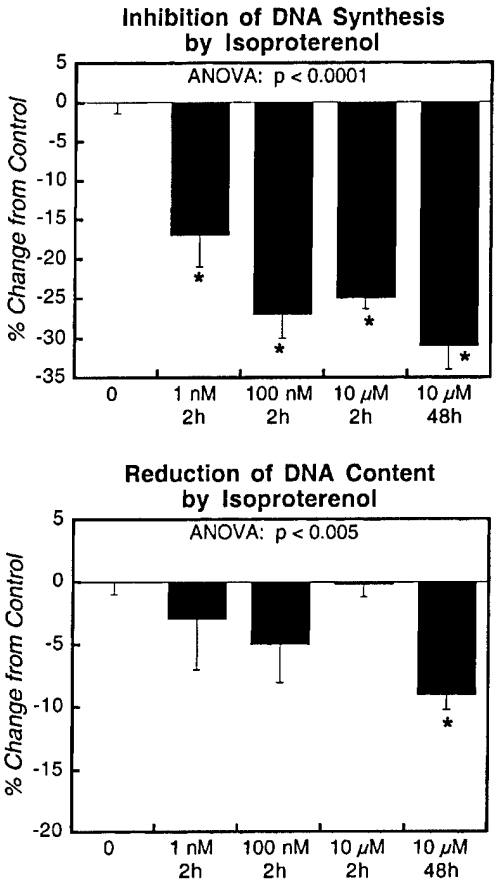

Figure 1. Effects of isoproterenol treatment on DNA synthesis and content, presented as the percentage change from control values. Data represent means and standard errors obtained from 12–96 determinations for each concentration and time point. Isoproterenol was added for 2 h or 48 h, with inclusion of [^{3}H]thymidine for the final hour. ANOVA across all treatments appears at the top of each panel and asterisks denote individual treatments that differ significantly from the control.

cells were pretreated for 48 h with dexamethasone and then received a 2-h challenge with isoproterenol, the inhibitory effects on DNA synthesis were less than additive: the net effect on DNA synthesis was no greater than that of isoproterenol alone, and the effect on DNA content was not distinguishable from that seen with just the dexamethasone pretreatment. However, when both treatments were combined for 48 h, the net effects on DNA synthesis and DNA content were greater than those achieved by either treatment alone, albeit less than additive.

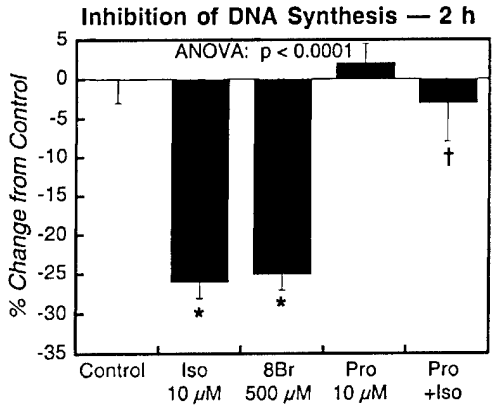

Figure 2. Effects of a 2-h treatment with isoproterenol (Iso), 8-bromo-cAMP (8Br), or propranolol (Pro) on DNA synthesis, presented as the percentage change from control values. Data represent means and standard errors obtained from 10–26 determinations for each concentration and time point. ANOVA across all treatments appears at the top of each panel; asterisks denote individual treatments that differ significantly from the control and the dagger denotes a significant difference between Pro + Iso and Iso alone. In addition, two-factor ANOVA for the effects of propranolol on the isoproterenol response indicated complete blockade ($p < 0.002$ for the main effect of isoproterenol, $p < 0.02$ for the main effect of propranolol, $p < 0.05$ for the interaction of the two treatments). None of the treatments produced a significant change in DNA content (data not shown).

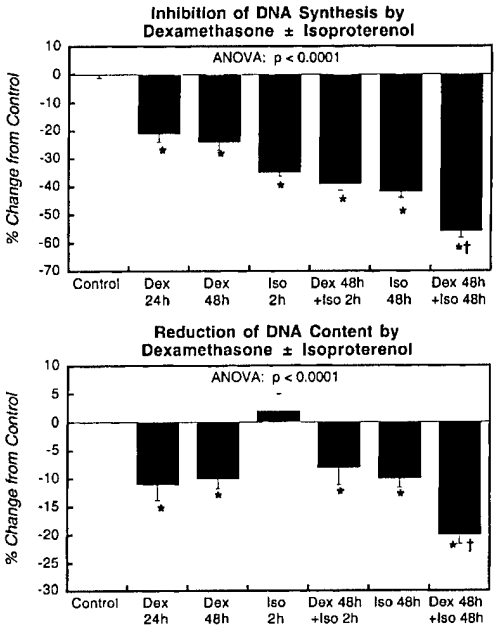

Figure 3. Effects of dexamethasone (Dex) alone or in combination with isoproterenol (Iso), on DNA synthesis and content, presented as the percentage change from control values. Data represent means and standard errors obtained from 11–54 determinations for each treatment. ANOVA across all treatments appears at the top of each panel; asterisks denote individual treatments that differ significantly from the control and the daggers denote significant differences between Dex 48 h + Iso 48 h, and the corresponding treatments with Dex or Iso alone. In addition, for DNA synthesis, two-factor ANOVA (dexamethasone 48 h × isoproterenol 2 h) indicates significant main effects of dexamethasone ($p < 0.0005$) and isoproterenol ($p < 0.0001$) but no interaction between the two treatments; with 48 h of both dexamethasone and isoproterenol treatment, there were significant main effects of both treatments ($p < 0.0001$ for each) as well as a significant interaction of dexamethasone × isoproterenol ($p < 0.002$). For DNA content, two-factor ANOVA (dexamethasone 48 h × isoproterenol 2 h) indicates a significant main effect of dexamethasone ($p < 0.009$) but no effect of isoproterenol; with 48 h of both dexamethasone and isoproterenol treatment, there were significant main effects of both treatments ($p < 0.0001$ for each).

In addition to measurements of DNA content, drug effects on the number and morphological characteristics of MDA-MB-231 cells were examined (Figures 4 and 5). In control cultures, cell morphology and size were essentially identical to those reported for the origination of the MDA-MB-231 line [32]. Sustained isoproterenol treatment reduced the total number of cells by over 20% and a comparable effect was seen for dexamethasone. Combined treatment with dexamethasone and isoproterenol had a comparably greater effect (30%), albeit not equivalent to the summation of the two individual effects. In order to maintain cAMP levels at the highest possible value, we also treated the cells with the phosphodiesterase inhibitor theophylline, with or without isoproterenol (Figure 4). Theophylline completely arrested mitosis, so that addition of isoproterenol had no further effect. Direct morphological examination (Figure 5) also confirmed that isoproterenol and dexamethasone, alone or in combination, reduced the number of cells. Again, theophylline caused massive reductions in cell number but in this case there were radical changes in cell morphology. The remaining cells were considerably larger than in the control group, indicating that the treatment did not prevent postmitotic cell growth and in addition, the cells assumed a distinct stellate appearance.

To determine whether the effects of isoproterenol are shared by all cancer cells expressing β-adrenoceptors, we compared the effects on MDA-MB-231 cells with those on rat C6 glioma cells (Figure 6). In contrast to the human breast cancer cells, C6 cells showed neither inhibition of DNA synthesis nor a reduction in DNA content over comparable periods.

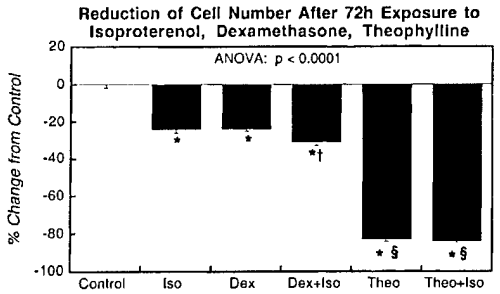

Figure 4. Effects of a 72-h treatment with isoproterenol (Iso), dexamethasone (Dex), or theophylline (Theo) on cell number, presented as the percentage change from control values. Data represent means and standard errors obtained from 10–22 determinations for each treatment. ANOVA across all treatments appears at the top of each panel. Asterisks denote individual treatments that differ significantly from the control; the dagger denotes a significant difference between Dex + Iso and either treatment alone; § denotes significant differences between theophylline with or without isoproterenol, as compared to all other treatments. In addition, two-factor ANOVA across the dexamethasone and isoproterenol treatments indicates significant main effects of each treatment alone ($p < 0.0001$) as well as a significant interaction of dexamethasone × isoproterenol ($p < 0.03$). Across the theophylline and isoproterenol groups, there were significant main effects of each treatment alone ($p < 0.0001$) as well as a significant interaction of theophylline × isoproterenol ($p < 0.0001$).

The maintenance of isoproterenol-induced inhibition of DNA synthesis in MDA-MB-231 cells over a 48-h span of continuous treatment suggested that either agonist-induced receptor downregulation or desensitization were not present in these cells, or alternatively, that stimulation of only a small number of receptors was sufficient to inhibit mitosis. Receptor downregulation can be selective for different subtypes and accordingly, we first evaluated which subtype was present in MDA-MB-231 cells. Using the β_1-selective antagonist CGP20712A, we found that displacement of [^{125}I]iodopindolol involved a single class of sites displaying an IC_{50} in the μM range (Figure 7). For contrast, we prepared cardiac cell membranes from one day old rats [27], which display predominance of the β_1-subtype [33]; in this preparation, CGP20712A displayed two IC_{50} values, one in the nM range corresponding to the major cardiac receptor population, and a minor component which, like the MDA-MB-231 cells, displayed an IC_{50} in the μM range. Accordingly, the subtype expressed by MDA-MB-231 cells is almost exclusively β_2.

We next determined whether isoproterenol treatment of MDA-MB-231 cells causes β_2-receptor downregulation and/or uncoupling of the receptors from their ability to stimulate adenylyl cyclase. In untreated cells, adenylyl cyclase activity declined by over 50% during the span of log-phase replication (Figure 8). However, the adenylyl cyclase response to isoproterenol fell by a significantly smaller proportion than did any of the other measures and the concentration of β-receptors was maintained at the same level throughout replication and growth. Relative to total cyclase catalytic activity (Mn^{2+}), the isoproterenol response actually increased over the course of culturing. After 1 day in culture, isoproterenol evoked 75 ± 3% of the total catalytic response exemplified by Mn^{2+}, whereas after 3–4 days in culture, the two stimulations were indistinguishable: isoproterenol evoked 97 ± 3% of the total response ($p < 0.0001$ compared to the proportion after 1 day in culture).

Despite the fact that isoproterenol-induced inhibition of DNA synthesis was maintained throughout a 48-h drug exposure, receptor downregulation was apparent immediately upon introduction of the drug (Figure 9). A concentration-dependent reduction in receptor binding was evident within 1 h, with nearly complete downregulation by 24 h. Receptor binding then remained at 5–10% of control values throughout 72 h of exposure. Isoproterenol concentrations as low as 1 nM produced significant, albeit submaximal, reductions in receptor binding after 24 h of exposure. Scatchard analysis confirmed that the loss of receptor binding reflected a decrease in the number of receptors as measured by maximal binding, rather than a change in receptor affinity as monitored by the K_d. In additional studies, we found that a 2-h daily isoproterenol exposure was sufficient to cause full receptor downregulation. We treated cells for three days in succession, using 100 μM isoproterenol for 2 h each day, followed in each case by 22 h without drug; 22 h after the third day's exposure, receptor binding was only 6 ± 1% of control values ($n = 6$, $p < 0.0001$). Similarly, even when we reduced the concentration to 1 μM with exposure for 2 h per day over a 2-day span, receptor measurements made 22 h after the last exposure still indicated robust downregulation (8 ± 1% of control, $n = 6$, $p < 0.0001$).

We also determined whether agonist-induced receptor downregulation was accompanied by loss of the adenylyl cyclase response to isoproterenol (Figure 10). Treatment of cells with 1 μM isoproterenol for 2 h, which caused approximately a 25% reduction in β-receptor binding, also elicited a comparable loss of the membrane response of adenylyl cyclase to isoproterenol. However, changes at the level of

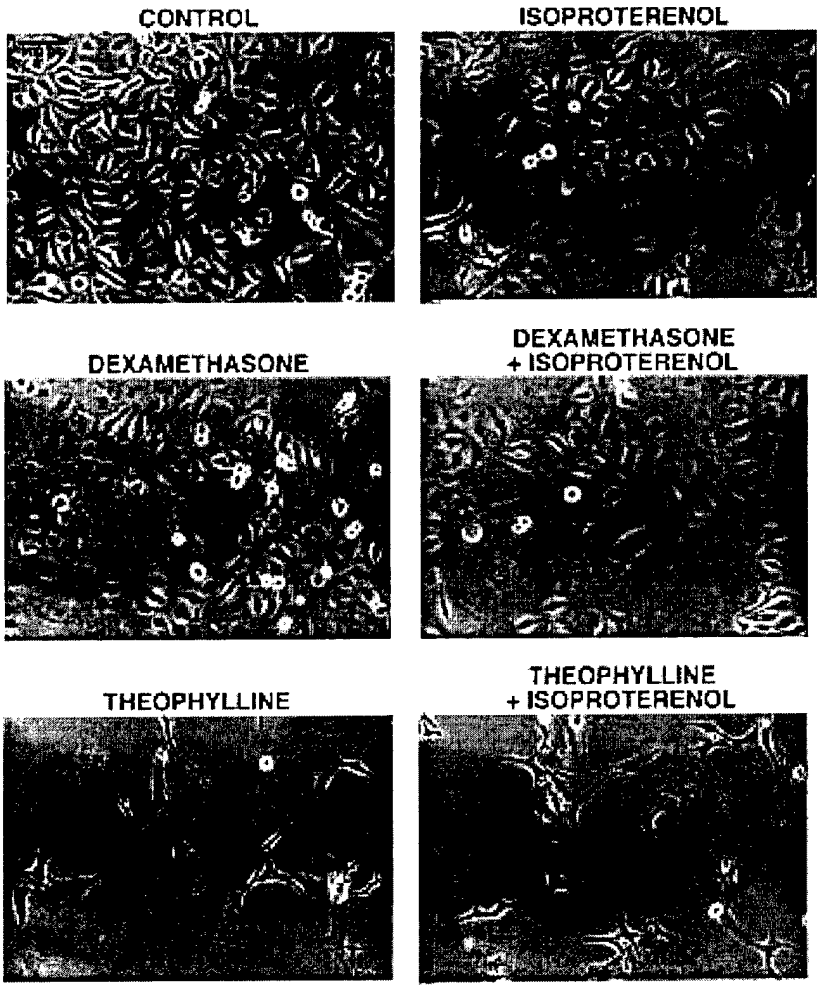

Figure 5. Phase-contrast microscopic appearance of MDA-MB-231 cells after a 72-h treatment with isoproterenol, dexamethasone, or theophylline. Scale bar appears in the upper left panel.

G-protein function were evident: basal enzyme activity, measured in the presence of GTP, also showed significant and immediate reductions, and the response to maximal G-protein activation by fluoride was impaired by a small amount. After a 48-h exposure to isoproterenol, desensitization of the membrane adenylyl cyclase response to isoproterenol reached 75%, not quite as large as the degree of receptor downregulation; again, effects on basal adenylyl cyclase activity also were present, but were not as notable as the change in the β-receptor-mediated response. At no point did we note any decline in the expression or catalytic activity of adenylyl cyclase itself, as monitored by the effect of Mn^{2+}.

In light of the enhanced effect on cell replication of the combination of dexamethasone and isoproterenol treatment, we also examined their interaction at the levels of β-adrenoceptor binding and adenylyl cyclase activity. Pretreatment of cells with dexamethasone for 24 h had no effect on β-receptor binding (Figure 11, top panel). However, when the pretreatment was combined with a subsequent 4-h exposure to isoproterenol, it enhanced the downregulation caused by the receptor agonist. Dexamethasone

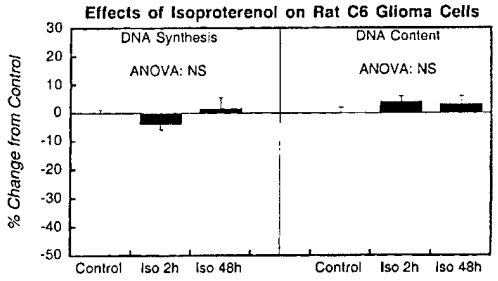

Figure 6. Effects of 10 μM isoproterenol (Iso) on DNA synthesis and content in rat C6 glioma cells, presented as the percentage change from control values. Data represent means and standard errors obtained from 11–30 determinations for each time point. ANOVA across all treatments appears within each panel.

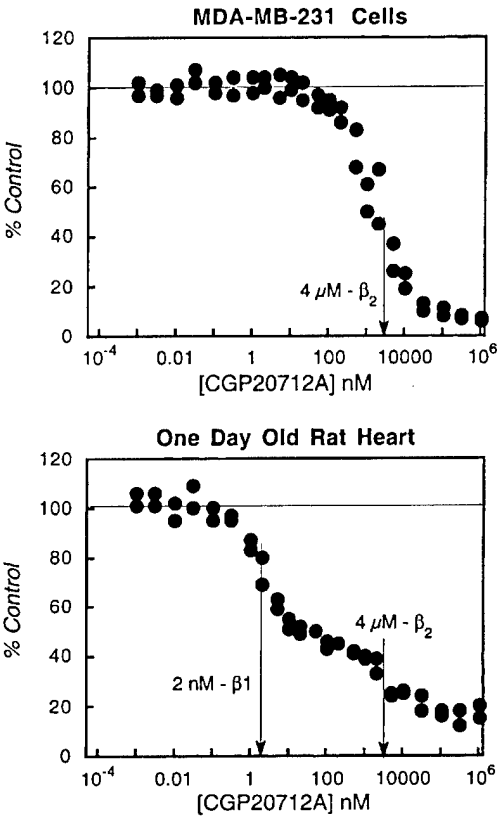

Figure 7. Binding of [^{125}I]iodopindolol to β-adrenoceptors in cell membranes prepared from MDA-MB-231 cells and from one day old rat heart, and its displacement by the β_1-specific antagonist CGP20712A. Each data point represents an individual determination, shown as the percentage of values obtained in the absence of displacer. The [^{125}I]iodopindolol concentration was 67 pM.

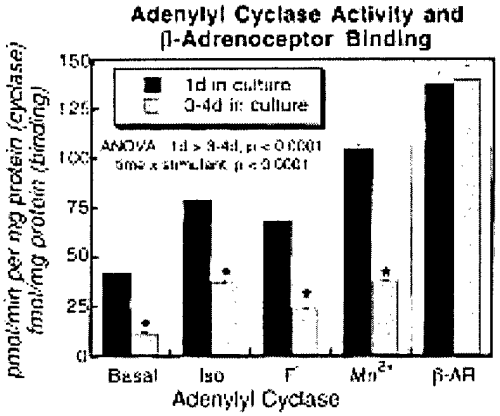

Figure 8. Adenylyl cyclase activity and β-adrenoceptor binding in MDA-MB-231 cells during cell replication and growth in culture. Cells were cultured for the indicated time periods and then membranes were isolated and enzyme activity determined under basal conditions or in the presence of 100 μM isoproterenol (Iso), 10 mM fluoride (F^-) or 10 mM Mn^{2+}. Data represent means and standard errors obtained from 6–24 determinations for each measurement at each time point. ANOVA across both time points and all stimulants appears within the panel and asterisks denote measures for which the values after 3–4 days in culture are significantly lower than the initial values. In addition, the cyclase response to isoproterenol declines less than the response to other stimulants ($p < 0.0001$).

had only small effects on adenylyl cyclase activity (Figure 11, bottom panel). By itself, dexamethasone lowered the membrane response to isoproterenol by a few percent. When dexamethasone pretreatment was superimposed on short-term isoproterenol treatment of the cells, basal adenylyl cyclase activity was inhibited slightly less than with isoproterenol alone and the fluoride response was inhibited somewhat more. However, the agonist-induced desensitization of the specific response to isoproterenol was just as prominent after dexamethasone pretreatment as it was without pretreatment.

Discussion

Among the epithelial, endocrine, and secretory cancer cell lines that express β-adrenoceptors, MDA-MB-231 human breast cancer cells exhibit comparatively high concentrations [13, 17, 26, 34, 35]. The B_{max} found here, 350 fmol/mg protein, exceeds the receptor concentration found in typical noradrenergic target tissues by over an order of magnitude [36] and is similar to the level found in brain regions enriched in noradrenergic projections. It is thus of critical

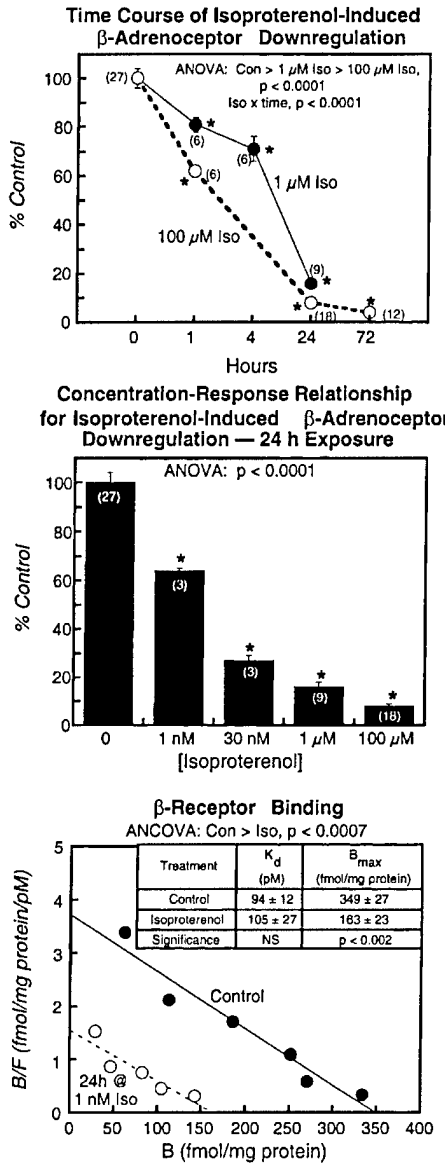

Figure 9. β-Adrenoceptor downregulation caused by isoproterenol treatment. Data represent means and standard errors obtained from the number of determinations shown in parentheses, determined as the percentage change from control values. In the top and middle panels, ANOVA across all time points or treatments appears at the top and asterisks denote individual values that differ significantly from the control. In the bottom panel, ANCOVA appears for the overall differences between Scatchard plots in control and isoproterenol-treated (Iso) cells.

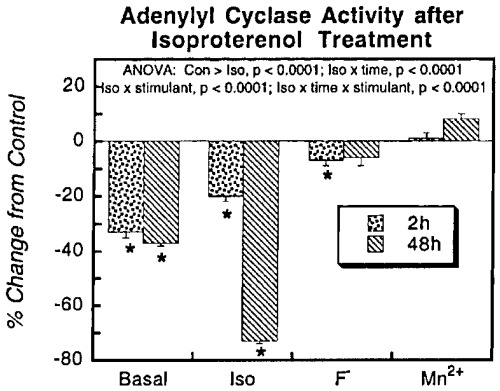

Figure 10. Desensitization of adenylyl cyclase evoked by exposure to 1 μM isoproterenol. Cells were treated with isoproterenol for the indicated time period and then membranes were isolated and enzyme activity determined under basal conditions or in the presence of 100 μM isoproterenol (Iso), 10 mM fluoride (F^-), or 10 mM Mn^{2+}. Data represent means and standard errors obtained from 6–12 determinations at each time point, determined as the percentage change from control values. ANOVA across all stimulants and both time points appears at the top and asterisks denote individual values that differ significantly from the control. Two-factor ANOVAs (treatment × time) were also assessed for each variable. Across both time points, basal activity was significantly reduced ($p < 0.0001$ for the main effect of isoproterenol), the response to isoproterenol was reduced in a time-dependent fashion ($p < 0.0001$ for the main effect, $p < 0.0001$ for the treatment × time interaction), the response to fluoride was reduced ($p < 0.0001$ for the main effect), and the response to Mn^{2+} was unaffected.

interest that stimulation of these receptors leads to immediate inhibition of DNA synthesis and, with prolonged exposure, reductions in the total number of cancer cells; inhibition of DNA synthesis is a reliable predictor of chemotherapeutic responses in breast cancer cells [37]. β-adrenoceptor-mediated inhibition of DNA synthesis was not shared by another cancer cell line, C6 rat glioma, that expresses a different β-receptor subtype at lower levels; whereas we found that MDA-MB-231 cells express β_2-receptors exclusively, C6 cells express primarily the β_1-subtype [38], but it is not clear whether this is the sole determinant of the role of β-adrenoceptors in the control of mitosis. Although there has been no systematic screening of breast cancer cell lines for β-adrenoceptor expression, these receptors have been identified in both estrogen-dependent and estrogen-independent types, including CG-5, BT-20, T47-D, VHB-1, and MCF-7 [13, 17, 26], as well as in tumors induced by administration by dimethylbenz(*a*)anthracene [34] and in unspecified collections of human breast cancer tissue samples [35]. In the two cases where receptor subtypes have

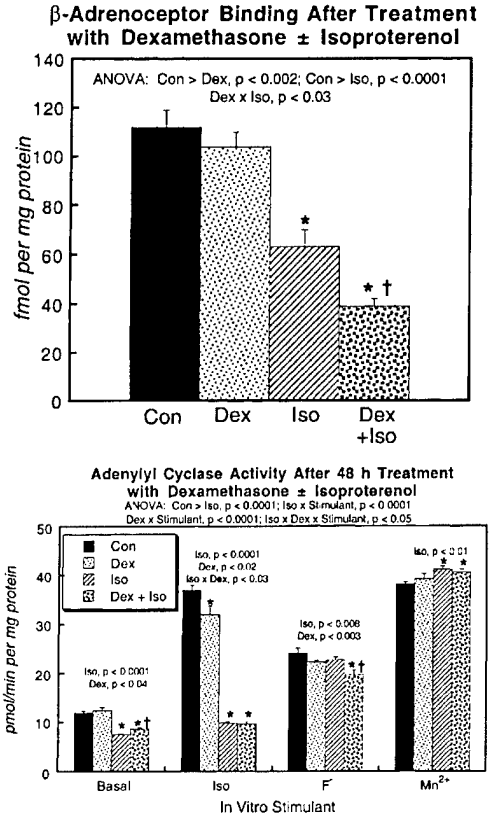

Figure 11. Effects of dexamethasone (Dex) alone or in combination with isoproterenol (Iso), on β-adrenoceptor binding (top) and adenylyl cyclase activity (bottom). Cells were pretreated with 10 μM dexamethasone for 24 h (top) or 48 h (bottom) and were then exposed to 1 μM isoproterenol for 4 h. For adenylyl cyclase, enzyme activity was determined under basal conditions or in the presence of 100 μM isoproterenol (Iso), 10 mM fluoride (F^-), or 10 mM Mn^{2+}. Data represent means and standard errors obtained from 6–12 determinations for each treatment. For receptor binding, ANOVA across all treatments appears at the top; for adenylyl cyclase, ANOVA across all treatments and *in vitro* stimulants appears at the top, and lower order ANOVAs are shown for *in vitro* stimulant with each cluster of bars. Asterisks denote individual treatments that differ significantly from the control, and the daggers denote significant difference of combined treatment from the effects of dexamethasone or isoproterenol alone.

been identified, the β_2-subtype appears to predominate, just as found here [13, 35], although unlike our findings with MDA-MB-231 cells, some cell lines also make significant amounts of β_1-receptors [13]. In the CG-5 cell line, which expresses both the receptor subtypes, but at lower concentrations than those found here for MDA-MB-231 cells, administration of a β-agonist evokes a biphasic response, with enhanced cell replication at low concentrations and inhibition at high concentrations. Nevertheless, it is generally the case that increased cAMP, whether induced by receptor activation or by addition of membrane-permeable cAMP analogs, inhibits cell replication and evokes tumor regression [17, 35] and that β-receptor expression is predictive of the overall pharmacologic response [34]. Accordingly, an understanding of the specific role of β-adrenoceptor function in different breast cancer cell lines will require evaluation of receptor concentration, subtype, the efficiency of coupling to the generation of cAMP, and, as evaluated here, the persistence of receptor activity in the face of agonist-induced receptor downregulation and desensitization.

Isoproterenol-induced inhibition of DNA synthesis in the MDA-MB-231 breast cancer cell line exhibited the characteristic properties of β-receptor actions mediated through adenylyl cyclase: complete blockade by propranolol, and sharing of the effect by a membrane-permeable cAMP analog. What was unexpected, however, was the fact that isoproterenol's effect did not disappear with prolonged treatment, and in fact, was maintained at exactly the same level as the initial effect. Ordinarily, β-adrenoceptor agonists elicit downregulation and desensitization over a time frame of a few minutes to hours [22], and therefore a loss of effect was expected here. The first hint of atypical regulation of β-receptor signaling was provided simply by monitoring adenylyl cyclase responses under control conditions. Over a course of several days in culture, adenylyl cyclase activity declined, accompanied by a loss of responsiveness to stimulants acting either at the level of β-receptors (isoproterenol), G-proteins (fluoride), or cyclase itself (Mn^{2+}). However, the enzymatic response to isoproterenol showed a smaller decline than for any other stimulant, that is, the β-adrenoceptor response actually increased relative to the total amount of catalytic activity. In fact, after 3–4 days in culture, isoproterenol elicited the maximal possible activation of adenylyl cyclase, since the activity was indistinguishable from that seen with addition of Mn^{2+}. In addition, the response to isoproterenol, which selectively activates G_s, was significantly greater than that to fluoride, which causes activation of both stimulatory and inhibitory G-proteins. The profound response to isoproterenol is unusual, since in most tissues, isoproterenol is incapable of eliciting adenylyl cyclase activation equaling that of direct G-protein or cyclase stimulants [23, 24, 30, 39]. The number of β-receptors also kept pace with cell divi-

sion and growth, so that the overall concentration of receptors remained unchanged throughout 4 days in culture, a period in which the number and size of cells increased substantially.

Given the maintenance of the ability of isoproterenol to inhibit DNA synthesis, we expected to see failure of receptor downregulation and/or desensitization, paralleling the situation during differentiation of normal cells possessing these receptors [23, 24, 30]. Surprisingly, isoproterenol caused immediate and robust receptor downregulation, accompanied by a parallel loss of the ability of receptor activation to stimulate adenylyl cyclase activity. Downregulation reached over 90% within 24 h and the adenylyl cyclase response was desensitized by 75% throughout the period in which inhibition of DNA synthesis was maintained. These results thus indicate that downregulation and desensitization do occur, but that the ability of receptor stimulation to inhibit DNA synthesis and cell acquisition requires activation of only a very small number of receptors. This raises the possibility that there may be adaptations in the signaling pathway downstream from receptors, G-proteins, and cyclase that serve to maintain the net effect of receptor stimulation. In keeping with this view, we have found that, during brain development, the initial stimulation of β-receptors 'programs' cAMP response elements so as to preserve or enhance the response of gene expression to adrenergic input [10, 39]. If similar events occur in MDA-MB-231 cells, then adaptations of downstream elements may preserve the effects on cell replication in the face of receptor downregulation and desensitization. Accordingly, a logical next step is to look at transcription factors and genes targeted by β-receptor stimulation and their role in the antimitotic effect of isoproterenol.

From both the standpoints of mechanism and therapeutics, our findings of augmented effects with cotreatment of dexamethasone or theophylline are potentially important. Initially, we expected dexamethasone to enhance the response to isoproterenol because glucocorticoids induce the synthesis of β_2-receptors in normal cells [40], especially during cell differentiation [27]. Dexamethasone treatment by itself inhibited DNA synthesis in MDA-MB-231 cells leading eventually to a reduction in cell number. In addition, when dexamethasone was combined with isoproterenol treatment, we saw even greater inhibition of DNA synthesis and loss of cells. However, when we examined the mechanism underlying the combined effect, we found that dexamethasone was not capable of preventing agonist-induced β-receptor downregulation or desensitization; in fact, the combination of dexamethasone and isoproterenol produced a more-than-additive receptor downregulation. The disparity between the augmented effects of combined dexamethasone and isoproterenol treatment on DNA synthesis, which were less than additive, and effects directed toward receptor expression, which were synergistic and in a direction opposite to that expected from the effects on cell replication, indicate that dexamethasone must be acting on elements downstream from the receptor. Indeed, earlier work in replicating and differentiating cells indicates that glucocorticoids induce G-proteins and adenylyl cyclase [27]. In the current study, we found significant increases in total adenylyl cyclase activity (i.e. enhanced response to Mn^{2+}) after combined treatment with isoproterenol and dexamethasone; although dexamethasone and isoproterenol individually produced receptor uncoupling from adenylyl cyclase, dexamethasone did not augment the uncoupling caused by isoproterenol, even in the face of greater receptor downregulation. Obviously, post-receptor targets, including but not limited to adenylyl cyclase, play a pivotal role in maintaining the net response to receptor stimulation in the face of extensive downregulation. Additional downstream factors, such as protein kinase A and phosphorylated target proteins, are necessary to couple β-adrenoceptors to effects on cell replication and differentiation, and future studies will need to determine how glucocorticoids alter their expression and/or function to maintain the anti mitotic response to receptor stimulation.

The second cotreatment that we examined was the phosphodiesterase inhibitor, theophylline. This drug alone had the greatest effect on mitosis: after several days in culture, the number of cells appeared to be no greater than that originally plated. Theophylline has a greater effect than isoproterenol because it interrupts the ability of phosphodiesterase to limit the rise of intracellular cAMP levels. Indeed, the effects of theophylline are precisely the same as those seen for effects of prolonged elevations of cAMP during normal cell development, namely termination of cell division in favor of cell growth and differentiation [5, 41, 42], the same type of effects seen for cAMP in several other cancer cell lines [16, 19]. Accordingly, the theophylline-treated cells also showed gross enlargement as well as a morphological change to a stellate appearance, confirming the separability of effects on mitosis from those on cell growth and differentiation.

In light of the findings for isoproterenol and dexamethasone, it is again possible that theophylline may influence gene expression downstream from cAMP generation, or alternatively, may act through other surface receptors (e.g. adenosine receptors) whose expression has not been explored in these cell lines.

Regardless of the ancillary mechanisms involved in β-adrenoceptor-mediated inhibition of mitosis in MDA-MB-231 breast cancer cells, the fact that inhibition does not disappear with receptor downregulation and desensitization raises the possibility for therapeutic strategies employing receptor agonists, alone or in combination with glucocorticoids and phosphodiesterase inhibitors. The cell line studied here, for example, is estrogen-insensitive and is thus nonresponsive to standard antiestrogen therapies. Furthermore, interventions operating at the level of cell surface receptors, such as β-adrenoceptors, do not require penetration of drug to the interior of the cell, and thus would not be subject to loss of effect from induction of transporters in multidrug resistance. Although theophylline was more effective than isoproterenol in reducing the number of cells in culture, it is distinctly possible that it would prove less effective with prolonged treatment *in vivo*. If theophylline acts through inhibition of phosphodiesterase, an intracellular locus, the cells may develop resistance, whereas if its actions are directed toward cell surface receptors (e.g. adenosine receptors), theophylline should maintain its effectiveness. From a mechanistic standpoint, input from receptors on the cell surface, like β-adrenoceptors, is 'upstream' from genes whose mutations lead to constitutive activation and cell proliferation [10, 43, 44]; receptor stimulation could thus limit the net effects of adverse mutations on cell cycle control by restricting expression of these genes. Given that desensitization effectively terminates the physiological effects of β-agonists in normal cells, therapeutic interventions based on receptor targeting should have only short-term side effects relative to the maintenance of effect in the target cell population. The concentrations of isoproterenol found to elicit inhibition of DNA synthesis and reductions of cell number in the current study (nM–μM) lie well within the range compatible with β-adrenergic effects in a wide variety of *in vivo* and *in vitro* systems [45] and approximates K_d values of isoproterenol for typical β-adrenoceptors [46, 47]. Consequently, it may be feasible to produce effective β-agonist concentrations for *in vivo* application; furthermore, the pharmacokinetics and toxicity of these agents are well-established. Neurotransmitter-based therapeutic strategies should thus be explored in an *in vivo* model to establish the potential utility of this general approach. Indeed, similar work with neuropeptides targeting opioid receptors has established their ability to control cell growth and replication in colon cancer [48, 49]. In the current case, screening of human cancers for the presence of β-adrenoceptors, or other cAMP-linked neurotransmitter receptors, along with evaluation of the response of the cells to receptor agonists or antagonists, may establish new treatment strategies.

Acknowledgements

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Address for offprints and correspondence: T.A. Slotkin, Box 3813 DUMC, Department of Pharmacology & Cancer Biology, Duke Univ. Med. Ctr., Durham, NC 27710, USA; *Tel.:* (919)681-8015; *Fax:* (919)684-8197; *E-mail:* t.slotkin@duke.edu

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Report

Antimitotic and cytotoxic effects of theophylline in MDA-MB-231 human breast cancer cells

Theodore A. Slotkin and Frederic J. Seidler
Department of Pharmacology & Cancer Biology, Duke University Medical Center, Durham, NC, USA

Key words: adenylyl cyclase, cell replication, cyclic AMP, DNA synthesis, protein synthesis, theophylline, xanthine derivatives

Summary

A variety of cancer cell lines, including MDA-MB-231 human breast cancer cells, exhibit mitotic inhibition by cAMP. In earlier work, we found that the phosphodiesterase inhibitor, theophylline, reduced the number of cells and altered cellular morphology. In the current study, we evaluated the effects of theophylline on macromolecule synthesis and indices of cell viability. Theophylline evoked a concentration- and time-dependent decrease in DNA synthesis. However, the net decrease in cell number was greater than that predicted solely from mitotic arrest. Assessment of protein synthesis indicated a second effect of theophylline separable from that on DNA synthesis. This was confirmed by decreased cell viability and adhesion. Exposure of the cells to the phosphodiesterase inhibitor, IBMX, in concentrations that produced inhibition of DNA synthesis equivalent to that seen with theophylline, elicited a smaller reduction in cell number. Theophylline also evoked specific changes in the expression or function of membrane-bound adenylyl cyclase activity, effects that are likely to contribute to sustained reactivity of these cells to other cAMP-related inhibitors of cell proliferation, such as isoproterenol. The multiple pharmacologic properties of theophylline, producing mitotic inhibition, cytotoxicity and altered signaling in MDA-MB-231 cells, may provide insight into novel therapeutic strategies.

Abbreviations: ANOVA: analysis of variance; cAMP: cyclic adenosine-3′,5′-monophosphate; DMSO: dimethylsulfoxide; IBMX: 3-isobutyl-1-methylxanthine.

Introduction

The emergence of breast cancers that are estrogen-insensitive and that also have the capability of developing multidrug resistance emphasizes the need for the development of alternative therapies. One potential strategy is to target cell surface receptors that mediate increases in intracellular cAMP levels, an effect that, in many different cell types, leads to inhibition of cell replication and consequent tumor regression [1–3]. To a large extent, cAMP in tumor cells thus recapitulates the role of this second messenger in normal cell development, namely termination of cell division in favor of cell differentiation [4–8]. In a recent study [9], we found that replication of MDA-MB-231 human breast cancer cells, an estrogen-insensitive cell line, is inhibited by stimulation of β-adrenoceptors located on the cell membrane, and that the underlying mechanism is stimulation of adenylyl cyclase activity, resulting in increased intracellular cAMP levels. β-Adrenoceptors are expressed on a variety of cancer cell lines derived from epithelial or secretory tumors [7, 10–13], including a number of breast cancers [1, 2, 9, 11, 14–16]. Nevertheless, there are also many cell lines that either do not express β-adrenoceptors, express only small concentrations of the receptors, or that respond to receptor stimulation by enhanced cell replication rather than inhibition [1, 11, 17]. It might therefore be useful to explore mechanisms to raise intracellular cAMP levels that do not depend solely on the expression of β-adrenoceptors.

In our previous work with MDA-MB-231 cells [9], we compared the effects of β-adrenoceptor agonists with membrane-permeable cAMP analogs and

with theophylline, which inhibits phosphodiesterase, the enzyme that mediates cAMP breakdown. The potential anticancer actions of theophylline or related xanthines were proposed over a decade ago [18], and a number of studies have shown that theophylline reduces cell division in a number of different cancer cell lines [19–21]. Surprisingly, we found that theophylline had greater effects on mitosis and cell morphology in the MDA-MB-231 breast cancer line than did either a β-adrenoceptor agonist or cAMP analogs, suggesting extra effects of this xanthine derivative besides inhibition of phosphodiesterase. This view is reinforced by the dichotomy in the cAMP and theophylline responses of MCF-7 breast cancer cells, where cAMP enhances the mitotic response to prolactin, whereas theophylline blocks the response [22]. Indeed, although phosphodiesterase inhibitors, like theophylline, reduce cell replication via the increase in cAMP levels, they clearly affect cell differentiation through multiple mechanisms [23]. Theophylline has a number of important cellular actions other than phosphodiesterase inhibition, notably its activity toward adenosine receptors, and its ability to generate reactive oxygen species. Ordinarily, adenosine receptor stimulation is thought to enhance cancer cell replication by decreasing intracellular cAMP levels [24]. However, theophylline and its derivatives possess a mixture of adenosine agonist-antagonist properties that can contribute to antitumor activity, but that depend on the differentiation state of the target cells [25]. Since theophylline is a xanthine derivative, there is a further possibility of free radical generation via xanthine oxidase, with the formation of cytotoxic levels of nitric oxide and peroxynitrite [26]. Free radicals produced by xanthine oxidase also affect cancer cell adhesion [27] and elicit DNA strand breaks [28, 29].

In the present study, we have evaluated the effects of theophylline on MDA-MB-231 human breast cancer cells, concentrating on comparisons of antimitotic activity, cytotoxicity and cell adhesion characteristics, and have contrasted the effects of theophylline with 3-isobutyl-1-methylxanthine (IBMX), a derivative that also inhibits phosphodiesterase and generates free radicals, but that is relatively insensitive toward adenosine receptors [26].

Methods

MDA-MB-231 cells (Duke University Comprehensive Cancer Center, Durham, NC) were seeded at a density of 10^6 cells per 100 mm diameter dish and maintained in modified minimum essential medium containing Earle's salts, 5% fetal bovine serum, 2 mM glutamine, 100 IU/ml of penicillin, 0.1 mg/ml of streptomycin and 5 μg/ml of insulin (all from Gibco, Grand Island, NY). Cells were incubated with 7.5% CO_2 at 37°C and the medium was changed every 24 h. Cells were examined at 100× magnification for counting and morphological features. Each experiment was repeated several times with separate batches of cells, after an average of five passages. Each passage and preparation was verified for morphology and growth rate. Cell viability was verified by exclusion of trypan blue. Treatment effects were always compared against concurrent control cultures from the same passage and batch of cells. Except as otherwise indicated, all drugs were obtained from Sigma Chemical Co. (St. Louis, MO).

Macromolecule synthesis and DNA content

To initiate the measurement of DNA or protein synthesis, the medium was changed to include 1 μCi/ml of [^{3}H]thymidine (specific activity, 2 Ci/mmol; New England Nuclear, Boston, MA) or [^{3}H]leucine (140 Ci/mmol; New England Nuclear). Incubations were carried out for 1 h, after which the medium was aspirated and cells were harvested in 3.5 ml of ice-cold water. Duplicate aliquots of each sample were treated with 10% trichloroacetic acid (TCA) and sedimented at 1000 × *g* for 15 min to precipitate macromolecules and the resultant pellet was washed once with TCA and, for measurements of DNA synthesis, with 75% ethanol. The final pellet was then hydrolyzed with 1 M KOH overnight at 37°C, neutralized with HCl and the DNA was then precipitated with ice-cold 5% TCA and sedimented at 1000 × *g* for 15 min. The supernatant solution, containing the solubilized protein, was counted for [^{3}H]leucine incorporation. For measurement of radiolabeled DNA and for DNA content, the pellet from this final step was hydrolyzed in 5% TCA for 15 min at 90°C, resedimented, and an aliquot of the supernatant solution counted for [^{3}H]thymidine incorporation. Another aliquot was assayed for DNA spectrophotometrically by absorbance at 260 nm. Previous work has demonstrated quantitative recovery of macromolecules by these techniques [30]. Incorporation values were corrected to the amount of DNA present in each culture to provide an index of DNA synthesis per cell.

Adenylyl cyclase activity

The medium was removed and cells were washed once with ice-cold, calcium- and magnesium-free Earle's balanced salt solution. Fresh solution was added and the cells were scraped off the dish and sedimented at 40,000 × *g* for 15 min. The pellet was resuspended (Polytron, Brinkmann Instruments, Westbury, NY) in 10 mM $MgCl_2$, and 50 mM Tris (pH 7.4) and the homogenate was sedimented at 40,000 × *g* for 15 min. The pellets were dispersed with a homogenizer (smooth glass fitted with a Teflon pestle) in 250 mM sucrose, 1 mM EGTA, 10 mM Tris (pH 7.4). Aliquots of membrane preparation containing ≈ 20 μg protein [31] were then incubated for 30 min at 30°C with final concentrations of 100 mM Tris–HCl (pH 7.4), 10 mM theophylline, 1 mM adenosine 5′-triphosphate, 10 mM $MgCl_2$, 1 mg/ml bovine serum albumin, and a creatine phosphokinase-ATP-regenerating system consisting of 10 mM sodium phosphocreatine and 8 IU phosphocreatine kinase, and 10 μM GTP in a total volume of 250 μl. The enzymatic reaction was stopped by placing the samples in a 90–100°C water bath for 5 min, followed by sedimentation at 3,000 × *g* for 15 min, and the supernatant solution was assayed for cAMP using radioimmunoassay kits (Amersham Corp., Chicago, IL). Preliminary experiments showed that the enzymatic reaction was linear well beyond the assay time period and was linear with membrane protein concentration; concentrations of cofactors were optimal and, in particular, the addition of higher concentrations of GTP produced no further augmentation of activity. In addition to evaluating basal activity, the maximal total activity of the adenylyl cyclase catalytic unit was evaluated with the response to 10 mM $MnCl_2$ [32].

The contributions of G-protein-linked processes to adenylyl cyclase were evaluated in two ways. First, to determine the net G-protein-linked response of adenylyl cyclase activity with maximal activation of all G-proteins, samples were prepared containing 10 mM NaF [32]. Second, β-adrenoceptor-targeted effects mediated through the G-proteins were evaluated with 100 μM isoproterenol. The concentrations of all the agents used here have been found previously to be optimal for effects on adenylyl cyclase and were confirmed in preliminary experiments [32, 33].

Data analysis

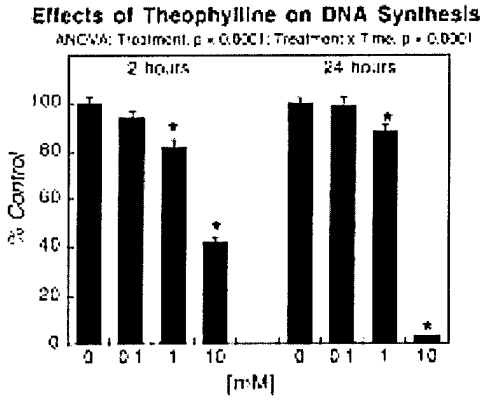
Data are presented as means and standard errors. For each study, treatment-related differences were first evaluated by a global ANOVA, incorporating all variables in a single test. For studies of adenylyl cyclase activity, multiple measurements were made from the same membrane preparation since several different stimulants were compared. In that case, stimulant was considered a repeated measure. Where significant treatment effects were identified with the global test, individual differences between treatment groups were established with Fisher's protected least significant difference. Significance for all tests was assumed at $p < 0.05$. For convenience, data are presented as a percentage of control values but statistical significance was always assessed on the unmanipulated data. Where multiple time points are presented in the same graph, the control groups are given as a single value (100%), but statistical comparisons were conducted only with the time-matched group appropriate to each treatment.

Results

As found previously [9], MDA-MB-231 cells grew in log-phase from 1 to 4 days after plating. The doubling time, based on cell counts and DNA content, was approximately 3 days, whereas the doubling of confluence was much quicker (1.5 days), indicating growth both by cell enlargement and replication (data not shown). Accordingly, drug treatments were initiated after one day in culture and were terminated at various times during the log-phase of growth.

Introduction of theophylline into the culture medium caused an immediate, concentration-dependent reduction in [^{3}H]thymidine incorporation into DNA (Figure 1). Significant inhibition was seen at 1 mM theophylline and inhibition was > 50% at 10 mM. After extended exposure (24 h), there was no change in the concentration threshold for inhibition of DNA synthesis but the effect at 10 mM increased to > 95% inhibition. Whereas there were no immediate effects of theophylline on cell number as monitored by DNA content, levels were 25% subnormal by 24 h after beginning exposure to the highest theophylline concentration; in an additional experiment conducted at 72 h, the DNA content in the theophylline-treated cells were reduced even further, to only 17 ± 1% of the control values ($P < 0.0001$, $n = 10$ for each group).

To determine whether the effects of theophylline on macromolecule synthesis were specific for DNA, we next determined whether similar treatment would



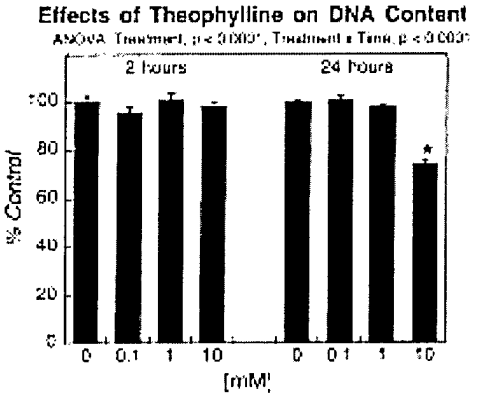


Figure 1. DNA synthesis and content after 2 or 24 h of theophylline exposure. Data represent means and standard errors obtained from 7–16 determinations at each concentration and time. Control [^{3}H]thymidine incorporation averaged 6000 dpm/μg DNA at 2 h and 5100 at 24 h; DNA content averaged 26 and 33 μg per dish, respectively. ANOVA across all concentrations and both time periods appears at the top of each panel. Asterisks denote individual values that differ from the corresponding control; in addition, the values at 10 mM theophylline are significantly different from those at 1 mM ($p < 0.0001$).

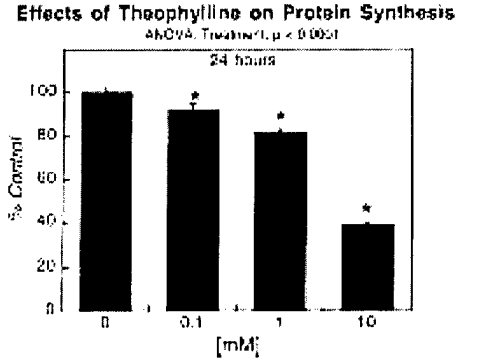


Figure 2. Effects of 10 mM theophylline on protein synthesis, measured after a 24 h exposure. Data represent means and standard errors obtained from eight determinations at each concentration. Control [^{3}H]leucine incorporation averaged 700 dpm/μg DNA. ANOVA across all concentrations appears at the top of the panel. Asterisks denote individual values that differ from the corresponding control; in addition, the values for each theophylline concentration are significantly different from each other ($p < 0.02$ or better).

affect protein synthesis (Figure 2). Twenty-four hours after beginning exposure to theophylline, there was a significant decrement in protein synthesis, with a lower threshold than had been seen for DNA synthesis: deficits were significant with as little as 0.1 mM theophylline. However, the maximal effect seen with 10 mM theophylline was not as great as for DNA synthesis: protein synthesis was reduced to 40% of control values, whereas inhibition of DNA synthesis was nearly total at that concentration and time. After 48 h of exposure to 10 mM theophylline, cell membrane protein was reduced by 50% (control, 0.62 ± 0.02 mg per dish; theophylline, 0.30 ± 0.01, $p < 0.0001$; $n = 6$).

The differences in the concentration-response curves for inhibition of DNA and protein synthesis suggested that theophylline might have additional effects besides antimitotic activity. Accordingly, we assessed cell number and viability after a 48-h exposure to 10 mM theophylline (Figure 3). Theophylline evoked nearly a 60% reduction in the total number of cells. Over and above the deficit in cell number, theophylline had adverse effects on viability of the remaining cells, as assessed by two indices, detachment and exclusion of trypan blue. The proportion of cells in each dish that were detached from the surface of the culture was nearly tripled. In addition, there was a doubling of the proportion of total cells (attached and detached) stained with trypan blue. Theophylline-induced cell detachment loss and of viability were related to each other. In control cultures, $5.3 \pm 0.3\%$ of the attached cells failed to exclude trypan blue, whereas $36 \pm 4\%$ of the detached cells were nonviable ($p < 0.0001$ versus attached cells). In the theophylline group, both these values were higher: $9.8 \pm 0.7\%$ nonviable attached cells ($p < 0.0001$ versus control group) and $55 \pm 2\%$ nonviable detached cells ($p < 0.0001$ versus control group; $p < 0.0002$ versus theophylline-exposed attached cells). Thus, over half of the detached cells in the theophylline group were

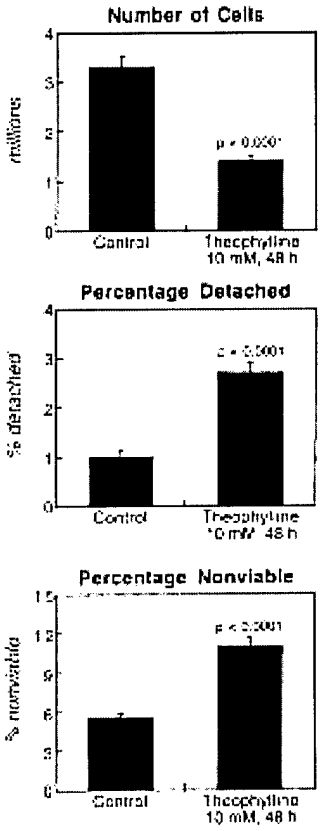


Figure 3. Cell number and viability after prolonged theophylline exposure. Data represent means and standard errors obtained from 16 determinations. Viability was determined as the percentage of total cells (attached and detached) that failed to exclude trypan blue. Detached cells were determined as the proportion of total cells (viable and nonviable) floating in the medium.

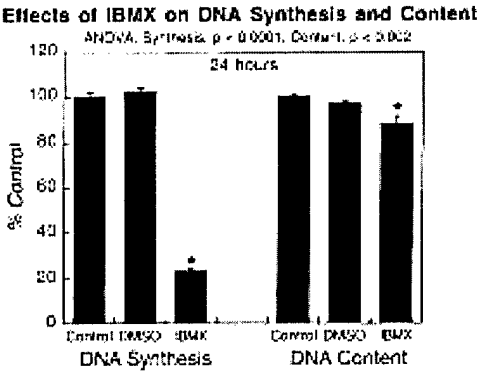


Figure 4. DNA synthesis and content after exposure to 1 mM IBMX. Data represent means and standard errors obtained from 10 determinations for each condition. As 0.1% DMSO vehicle was required to dissolve IBMX, values are compared for untreated cells (control), vehicle, and IBMX. ANOVA across all conditions appears at the top of the panel, and asterisks denote individual values that differ from the corresponding control or DMSO values.

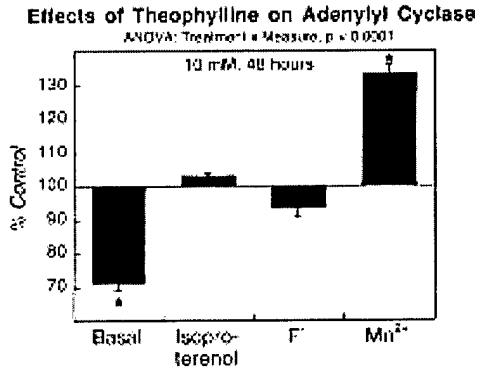


Figure 5. Effects of exposure to 10 mM theophylline on adenylyl cyclase activity measured under basal conditions, or with addition of 100 μM isoproterenol, 10 mM sodium fluoride, or 10 mM manganese chloride. Data represent means and standard errors obtained from six determinations for each stimulant. Control activities were: basal, 27 pmol/min per mg protein; isoproterenol, 70; fluoride, 59; manganese, 73. ANOVA across all measures appears at the top of the panel and asterisks denote individual values that differ from the corresponding control.

nonviable, whereas only a third of those in the control group were nonviable.

Theophylline possesses distinctly different pharmacological modalities, as a phosphodiesterase inhibitor, an adenosine receptor antagonist, and a xanthine. Therefore, we conducted similar studies using IBMX, a phosphodiesterase inhibitor with much lower activity toward adenosine receptors. As IBMX requires a solvent (0.1% DMSO) in order to dissolve in an aqueous medium, we compared untreated cells to cells exposed to DMSO alone, and to cells exposed to IBMX in DMSO vehicle (Figure 4). With a 24-h exposure, 1 mM IBMX evoked 80% inhibition of DNA synthesis, a much greater reduction ($p < 0.0001$) than that seen at the same concentration of theophylline (≈ 15% inhibition). Nevertheless, IBMX elicited only a 10% reduction in DNA content, no greater than that seen with the much less effective theophylline treatment. Indeed, when compared to 10 mM theophylline, a concentration that produced roughly equivalent inhibition of DNA synthesis to that caused by 1 mM IBMX, the effect of IBMX on DNA content was significantly less: 9 ± 3% reduction for IBMX

(Figure 4), 26 ± 1% reduction for theophylline (Figure 1; $p < 0.0001$ versus IBMX).

In our previous work [9], we showed that stimulation of cellular cAMP levels with a β-adrenoceptor agonist led to reductions in DNA synthesis, accompanied by desensitization at the level of receptor-mediated adenylyl cyclase activity. Accordingly, we evaluated whether theophylline, which inhibits cAMP breakdown, also targets the function of this signaling cascade. After a 48-h exposure to 10 mM theophylline, basal adenylyl cyclase activity was significantly reduced but signaling mediated by the β-adrenoceptor stimulant, isoproterenol, was maintained. Similarly, the response to fluoride, which evokes maximal stimulation of the G-proteins that link the receptors to adenylyl cyclase, was unaffected despite the lowering of basal enzyme activity. The response to manganese, which stimulates adenylyl cyclase directly, without the requirement for receptors or G-proteins, was significantly enhanced by theophylline treatment.

Discussion

In our previous work with MDA-MB-231 cells, we found that increasing the intracellular cAMP concentration led to a decrease in mitosis, so that membrane-permeable cAMP analogs, a β-adrenoceptor agonist (isoproterenol), or a phosphodiesterase inhibitor (theophylline), all led to a decrease in cell number [9]. However, theophylline was far more effective than any other treatment; at 2 h, 10 mM theophylline decreased DNA synthesis more than 50%, whereas maximally-effective concentrations of 8-bromo-cAMP produced only a 25% reduction [9]. Moreover, theophylline induced major morphologic changes not seen with the other agents [9] and the current results indicate that theophylline has collateral actions that affect cell number and viability. A comparison of the results of inhibition of DNA synthesis by theophylline, and the eventual reduction in cell number, provides the first indication of these additional effects. Based on the observed doubling time of MDA-MB-231 cells under our culture conditions, complete mitotic arrest for a 48 h span would result in a 35–40% deficit in total cell number. However, 10 mM theophylline caused a significantly greater reduction: at 48 h, the theophylline had only 40% of the control cell number; by 72 h, measurements of DNA content indicated a deficit of over 80%, whereas mitotic arrest predicts only a 50% deficit. The unexpectedly large deficit in cell number is even more impressive in light of the fact that theophylline did not cause complete mitotic arrest, but rather elicited only 60% inhibition in the initial exposure period. Indeed, the fact that theophylline's effects on DNA synthesis intensify over a 24-h span, implies that subsequent cellular changes are occurring that impact on cell division.

These results suggest that theophylline impacts events over and above the direct effect on cAMP and hence on cell replication. Our findings for protein synthesis and viability confirm this conclusion. Protein synthesis was reduced after a 24-h exposure to theophylline at concentrations that did not affect DNA synthesis; furthermore, the entire concentration-response curve for effects on protein synthesis differed from that on DNA synthesis, as the maximal effect toward protein was considerably less. After 48 h, cell membrane protein was reduced by 50%, approximately the same as the earlier inhibition of protein synthesis. However, cell number was decreased even more at 48 h (≈ 60%), indicating a rise in the membrane protein content per cell. This result is expected, given that theophylline produces a change in cell morphology, with development of cytoplasmic projections, with a consequent increase in membrane surface area [9].

The studies with cell attachment and trypan blue exclusion provide further evidence that theophylline has an effect on cell viability in addition to its effects on replication. Theophylline exposure more than doubled the proportions of detached cells and of non-viable cells. It is unlikely that increased cAMP levels consequent to phosphodiesterase inhibition, can solely account for these findings. When we compared the effects of theophylline with those of IBMX, another xanthine-based, phosphodiesterase inhibitor, we obtained results indicative of actions separable from those on cAMP. A concentration (1 mM) of IBMX that produced inhibition of DNA synthesis equivalent to that seen with 1–10 mM theophylline failed to evoke a decrease in cell number beyond what would have been expected from mitotic inhibition, a situation very different from the effects of theophylline.

There are two likely supplementary mechanisms for theophylline's actions on cell viability: formation of oxidative free radicals, and actions at adenosine receptors. Theophylline is a substrate for xanthine oxidase, an enzyme known to generate free radicals and resultant cell damage [26–29]. However, IBMX is also a xanthine and similarly induces free radical formation [26]. Thus, either theophylline is more potent than IBMX in eliciting oxidative stress, or alternatively,

it elicits cytotoxicity through collateral mechanisms, such as actions on adenosine receptors [25]. Indeed, free radicals produced by xanthine oxidase tend to increase cancer cell adhesion [27], whereas a decrease was seen with theophylline in MDA-MB-231 cells, implying that free radicals alone cannot account for all aspects of theophylline's cytotoxicity. The alternative, namely a role of adenosine receptors in cancer cell replication, differentiation and cytotoxicity has been explored only sporadically. Activation of the receptors reduces cell replication [24] and theophylline, acting as an antagonist, might then be expected to enhance, not inhibit mitosis. However, recent work indicates that under varying conditions of cell differentiation, theophylline derivatives possess mixed agonist-antagonist properties that can contribute to cytotoxicity [25]. It is thus likely that the effects of theophylline on MDA-MB-231 cells represents the summation of three different mechanisms: phosphodiesterase inhibition, free radical formation, and actions at adenosine receptors.

All three mechanisms are also likely to contribute to the distinct changes in cell morphology and function seen after theophylline exposure [9]. Oxidative stress itself induces cell differentiation [34]; since the increase in cAMP evoked by phosphodiesterase inhibition also serves to switch cells from replication to differentiation [4–9], the combined effect can account for the profound change in morphology evoked by theophylline but not by treatments that have more modest effects restricted only to cAMP [9]. The morphological changes elicited by theophylline suggest a profound change in differentiation state, and we obtained evidence for altered cell reactivity after theophylline exposure, another characteristic of differentiation. The adenylyl cyclase signaling cascade undergoes substantial changes during growth of MDA-MB-231 cells, characterized by a loss of basal enzyme activity but preservation of signaling mediated through membrane-bound β-adrenoceptors [9]. Treatment of the cells with theophylline similarly evoked a decrease in basal enzyme activity with maintenance of the response to stimulation of β-adrenoceptors by isoproterenol, or to fluoride-induced stimulation of the G-proteins that couple the receptors to adenylyl cyclase. Furthermore, there was specific enhancement of the response to manganese, which operates directly on adenylyl cyclase itself, without the participation of receptors or G-proteins; this implies either that adenylyl cyclase is induced by theophylline, or alternatively, that there is a shift toward expression of a more active isoform. In either case, the promotional effect on adenylyl cyclase is likely to augment the effects of theophylline on cAMP generation, enhancing the effect of phosphodiesterase inhibition. The induction of adenylyl cyclase and preservation of the β-adrenoceptor effect throughout differentiation may contribute additionally to the maintained ability of isoproterenol to inhibit MDA-MB-231 cell replication in the face of receptor downregulation that would ordinarily limit the response, thus augmenting the antitumor effect of other potential therapeutic interventions [9].

It is noteworthy that the concentration-response relationships for the various actions of theophylline span several orders of magnitude. Although the K_d for its actions on adenosine receptors lies in the micromolar range [35], its K_i for inhibition of phosphodiesterase and consequent effects on cAMP levels requires millimolar concentrations [36], and the potential requirements for generation of oxidative species remain to be explored. The clinical use of theophylline generally requires plasma concentrations no higher than 100 μM, below the concentration typical for phosphodiesterase inhibition, but within the threshold that we found necessary for inhibition of DNA and protein synthesis. Thus, if theophylline's actions on tumor growth were dictated solely by cAMP-dependent processes, the drug would have to be regarded as a prototype for design of more potent analogues targeting phosphodiesterase, an area of pursuit that has been well worked out [36]. However, it would be a mistake to focus solely on phosphodiesterase as the target for antitumor actions, given that our results clearly show participation of other processes, including but not limited to receptor targets and oxidative damage. Indeed, IBMX is a far more potent phosphodiesterase inhibitor [36] but proved to be less effective in inhibiting the growth of MDA-MB-231 cells, and it has been shown recently that xanthines can elicit apoptosis through actions on adenosine receptors, exclusive of cAMP-related effects [37]. Consequently, although a wide variety of drugs has been designed to target specific phosphodiesterase subtypes and to reduce activity toward adenosine receptors or xanthine oxidase, a better approach might be to optimize phosphodiesterase inhibition while augmenting actions at these other targets. In any case, the fact that we were able to target MDA-MB-231 cells, an estrogen-independent, and particularly aggressive tumor line, points out that xanthine-based therapeutic approaches may represent a viable alternative when hormone ant-

agonist therapy fails. Although there have been no systematic studies of anticancer effects of xanthine derivatives in hormone-dependent versus-independent cell lines, there is no *a priori* reason to suspect that the presence or absence of estrogen receptors is especially critical for these actions, and in one report with MCF-7, which is positive for the estrogen receptor, theophylline prevented stimulation of tumor growth by other factors [22]. Indeed, xanthines have been shown empirically to affect the growth of a variety of tumor lines other than breast cancers [18–21, 38], although to our knowledge, the current results are the first to show definitively that the cytotoxicity associated with theophylline's antitumor actions reflects multiple cellular targets beyond inhibition of phosphodiesterase. At the same time, the only suspected action of theophylline on healthy breast tissue is promotion of benign epithelial growths, and even this has been seriously challenged [39, 40]. Thus, the multiple pharmacologic properties of theophylline, producing mitotic inhibition, cytotoxicity and altered signaling in MDA-MB-231 cells, may provide insight into novel therapeutic strategies. In light of the antitumor effects seen here, it may be worthwhile to reexamine the potential chemotherapeutic use of xanthine derivatives.

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Address for offprints and correspondence: Dr. T.A. Slotkin, Box 3813 DUMC, Department of Pharmacology & Cancer Biology, Duke Univ. Med. Ctr., Durham, NC 27710 USA; *Tel.:* (919)681-8015; *Fax:* (919)684-8197; *E-mail:* t.slotkin@duke.edu

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Report

Generation of reactive oxygen species by xanthine derivatives in MDA-MB-231 human breast cancer cells

T.L. Crumpton, F.J. Seidler, and T.A. Slotkin
Department of Pharmacology & Cancer Biology, Duke University Medical Center, Durham, NC, USA

Key words: oxidative stress, reactive oxygen, sodium nitroprusside, theophylline, xanthine derivatives

Summary

Theophylline reduces cell number in MDA-MB-231 cells through mechanisms over and above phosphodiesterase inhibition. In the current study, we used an intracellular fluorescent dye to show that theophylline and, to a much greater extent, 3-isobutyl-1-methylxanthine, evoke the generation of reactive oxygen species and also sensitize the cells to insult by other oxidants. Xanthine derivatives may therefore offer novel strategies for antitumor therapeutics.

Abbreviations: ANOVA: analysis of variance; IBMX: 3-isobutyl-1-methylxanthine; ROS: reactive oxygen species

Introduction

Estrogen-insensitive breast cancers represent a major therapeutic problem, requiring the development of alternative strategies. Earlier studies with MDA-MB-231 human breast cancer cells [1, 2], found that theophylline arrests mitosis and elicits cytotoxicity through mechanisms over and above inhibition of phosphodiesterase, the enzyme that mediates cyclic AMP breakdown. Theophylline has antitumor activity in a number of different cancer lines [3–6] and in many cases there is a dichotomy between the responses to theophylline *versus* cyclic AMP [2, 7, 8].

Xanthine derivatives exhibit activity toward adenosine receptors as well as toward generation of reactive oxygen species (ROS) [9–13]. Accordingly, in the present study, we have evaluated the ability of theophylline and 3-isobutyl-1-methylxanthine (IBMX), a derivative that also inhibits phosphodiesterase and generates free radicals [10], to generate ROS in MDA-MB-231 cells.

Methods

MDA-MB-231 cells (Duke University Comprehensive Cancer Center, Durham, NC) were seeded at a density of 10^6 cells per 100 mm diameter dish and maintained in modified Minimum Essential Medium containing Earle's salts, 5% fetal bovine serum, 2 mM glutamine, 100 IU/ml of penicillin, 0.1 mg/ml of streptomycin and 5 μg/ml of insulin (all from Gibco, Grand Island, NY). Cells were incubated with 7.5% CO_2 at 37°C and the medium was changed every 2–3 days. Each experiment was repeated several times with separate batches of cells, after an average of five passages. Except where indicated, all drugs were obtained from Sigma Chemical Co. (St. Louis, MO).

Assay conditions were carried out as described in earlier work with PC12 cells [14]. Cells were harvested in Krebs-Ringer-bicarbonate buffer (120 mM NaCl; 5 mM KCl; 1 mM $MgSO_4$; 1 mM $CaCl_2$; 10 mM HEPES; 25 mM $NaHCO_3$; 1 mM NaH_2PO_4; 10 mM glucose) and sedimented at 250 × *g* for 10 min. The supernatant solution was removed and the remaining cells were washed twice with buffer and resedimented. The cells were counted and the cell concentration was adjusted to 6.5×10^5 per ml. An aliquot of cell suspension was loaded with 30 μM dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR) for 15 min in the dark, and then fluorescence was monitored for the ensuing 10 min with or without the addition of 1 mM theophylline or IBMX. Quantitation was based on the steady-state slope of

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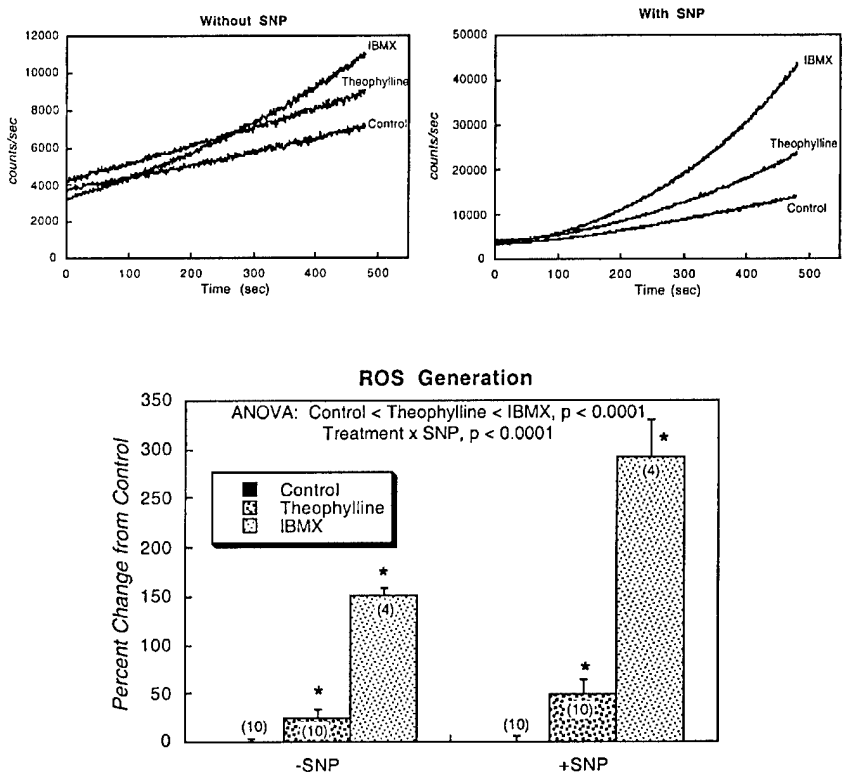

Figure 1. Generation of reactive oxygen species (ROS) in MDA-MB-231 cells. Cells were preloaded with dye and were then exposed to 1 mM theophylline or 3-isobutyl-1-methylxanthine (IBMX) for 10 min, either by themselves or concurrently with 0.3 mM sodium nitroprusside (SNP) challenge. The top panels show representative fluorescence determinations; note the different scale for samples to which SNP was added (right). The bottom panel shows the percent change from control values as means and standard errors, with the number of determinations shown in parentheses. ANOVA across all treatments appears within the panel, and asterisks denote individual values that differ significantly from the corresponding control. In addition, the effects of the drugs are significant when assessed separately with or without SNP ($p < 0.0001$ in both cases) and the effects of IBMX are significantly greater than those of theophylline ($p < 0.0001$ either with or without SNP).

fluorescence achieved by the end of the 10-min test period.

In addition to determining whether theophylline or IBMX lead to ROS generation, we also evaluated whether the drugs potentiated the oxidative response to 0.3 mM sodium nitroprusside (SNP), a compound that generates ROS directly and also through intracellular production of nitric oxide [14, 15]. SNP was added simultaneously with the drugs [14].

Results and discussion

Without addition of drugs, there was a small, spontaneous rate of oxidation of the intracellular dye; incubation with SNP produced a large increase in the generation of oxidative species (Figure 1). Addition of 1 mM theophylline caused a significant increase in ROS generation and also increased the response to SNP by the same proportion. An equimolar concentration of IBMX caused a much greater increase in ROS generation and in the SNP response.

Thus, there are two distinct effects of the xanthine derivatives contributing to oxidative stress in MDA-MB-231 cells. Both theophylline and IBMX evoke ROS formation by themselves, and in addition, they enhance the oxidative response to SNP, indicating that the xanthines sensitize the cells to other oxidative stressors. These results suggest that xanthine derivatives should be designed to emphasize oxidative responses; given the higher metabolic rate of cancer cells, tumor cells are likely to be especially targeted

for such actions, given that derivatives can be obtained which will enhance tumor selectivity and minimize normal tissue toxicities. As the xanthines sensitize the cells to other oxidative stressors, there is also an opportunity to design combination therapies targeting ROS formation.

Although IBMX is a far more potent phosphodiesterase inhibitor than theophylline [16] and also proved to be much more effective in generating ROS, we found previously that theophylline was at least as effective as IBMX in inhibiting growth of MDA-MB-231 cells [2], suggesting that a third class of xanthine effects may represent an additional therapeutic target. One possibility is the activation of adenosine receptors to elicit apoptosis [17]. In normal cells, this occurs through the A3 subtype, for which neither theophylline nor IBMX are particularly effective. Accordingly, if apoptosis occurs through adenosine receptor-mediated mechanisms in MDA-MB-231 cells, it would require either the presence of atypical receptors, or alternatively, activation of other receptor subtypes could be linked to apoptosis in these cells. In comparing phosphodiesterase inhibition, ROS formation and adenosine receptor actions, it is also important to note the differences in concentrations required for each effect. The K_d for theophylline's actions on adenosine receptors other than the A3 subtype lies in the μM range [18], whereas its K_i for inhibition of phosphodiesterase requires mM concentrations [2, 16]. Our current results indicate that ROS formation occurs at concentrations intermediate to the two other potential mechanisms. In clinical settings, theophylline exhibits significant side effects at concentrations above 100 μM, obviously below the concentration necessary for phosphodiesterase inhibition. However, cytotoxicity and inhibition of cell replication occur at the lower concentrations commensurate with adenosine receptor actions and possibly, as seen here, ROS generation [1, 2]. Therefore, in the discovery process for new antitumor agents it might be useful to pursue the design of xanthine derivatives emphasizing the latter two mechanisms as opposed to phosphodiesterase inhibition. In that regard, compounds resembling IBMX are likely to be much more effective than theophylline.

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Address for offprints and correspondence: T.A. Slotkin, Box 3813 DUMC, Department of Pharmacology & Cancer Biology, Duke University Medical Center, Durham, NC 27710 USA; *Tel.:* (919)681-8015; *Fax:* (919)684-8197; *E-mail:* t.slotkin@duke.edu

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β-ADRENOCEPTOR SIGNALING AND ITS CONTROL OF CELL REPLICATION IN MDA-MB-231 HUMAN BREAST CANCER CELLS

Theodore A. Slotkin, Jian Zhang, Ria Dancel, Stephanie J. Garcia, Carrie Willis and Frederic J. Seidler

Department of Pharmacology and Cancer Biology
Duke University Medical Center

t.slotkin@duke.edu

β-Adrenoceptors (βAR) are present on the cell surface in a number of different human breast cancer cell lines, including those that are nonresponsive to estrogen, and that are thus resistant to antiestrogen therapy. MDA-MB-231 human breast cancer cells express high βAR levels, predominantly the β_2 subtype. Receptor stimulation by isoproterenol evoked immediate reductions in DNA synthesis which were blocked completely by the receptor antagonist, propranolol. The effect was mimicked by administration of membrane permeable cyclic AMP analogs, and showed additive actions with glucocorticoids. With continued exposure, the effect of isoproterenol was maintained, despite βAR downregulation; indeed, receptor downregulation was evident with as little as 1h of isoproterenol treatment and over 90% of the receptors were lost within 24h. Receptor downregulation was paralleled by homologous desensitization of the adenylyl cyclase response to βAR stimulation. After 48-72 h of isoproterenol treatment, a significant reduction in cell number was obtained. Addition of a dexamethasone enhanced the loss of cells even though it did not prevent receptor downregulation or desensitization of adenylyl cyclase. Theophylline, which inhibits the breakdown of cyclic AMP, evoked complete mitotic arrest. These results indicate that β-adrenoceptors are effectively linked, through cyclic AMP, to the termination of cell replication in MDA-MB-231 human breast cancer cells, and that activation of only a small number of receptors is sufficient for a maximal effect. βAR-targeted therapy may offer a chance to slow the growth and spread of some estrogen nonresponsive breast cancers. Since the effects on the cancer cells do not desensitize, whereas effects on normal cells do, effective regimens may be designed that minimize side effects. Novel pharmacologic strategies that focus on cell surface receptors operating through adenylyl cyclase may offer opportunities to combat cancers that are unresponsive to hormonal agents, or that have developed multidrug resistance.

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