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Modulation of α_{1B}-adrenoceptor expression by agonist and protein kinase inhibitors

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Abstract

The agonist-induced up-regulation of α_{1B}-adrenoceptors in clone H99 of transfected Chinese hamster ovary cells that we reported previously (Zhu et al., 1996) was further investigated. Studies with a larger number of clones revealed that the up-regulation observed in H99 cells is atypical and that most other clones exhibit *down*-regulation under the same conditions. The role of protein kinases in the up-regulation of α_{1B}-adrenoceptors in clone H99 was further investigated. Surprisingly, the protein kinase inhibitor staurosporine induced a similar up-regulation. Neither the selective protein kinase C inhibitor GF109203X nor the activator phorbol 12-myristate, 13-acetate altered receptor expression. The tyrosine kinase inhibitors genistein and its weaker analog daidzein did not induce up-regulation but blocked the up-regulation induced by epinephrine and by staurosporine. Up-regulation was blocked by the protein synthesis inhibitor cycloheximide. These studies suggest multiple mechanisms by which different protein kinases can modulate the expression of transfected α_{1B}-adrenoceptors. © 1997 Elsevier Science B.V.

Keywords: α_{1B}-Adrenoceptor; Receptor up-regulation; Receptor down-regulation; Protein kinase; Staurosporine; Genistein

1. Introduction

Exposure of cells for more than a few hours to agonists acting at G-protein-coupled receptors, such as the adrenoceptor family, typically leads to a decrease in the number of cellular receptors, a phenomenon referred to as down-regulation. Down-regulation is a prominent feature in the overall process of agonist-induced desensitization of β_2-adrenoceptors, the most thoroughly characterized of the adrenoceptors (Toews et al., 1991; Kobilka, 1992; Lohse, 1993; Hadcock and Malbon, 1993). Down-regulation also occurs for α_{1B}-adrenoceptors, but the extent of down-regulation is generally less than that for β_2-adrenoceptors (Toews et al., 1991). Although the molecular mechanisms involved in down-regulation are not fully understood for any of the adrenoceptors, changes in gene transcription, receptor synthesis and receptor degradation are all likely to play a role in the regulation of receptor expression during long-term exposure of cells to drugs.

In preparation for site-directed mutagenesis studies of α_{1B}-adrenoceptor internalization and down-regulation, we characterized the regulation of the wild-type hamster α_{1B}-adrenoceptor transfected into Chinese hamster ovary (CHO) cells (Zhu et al., 1996). Surprisingly, in the clone chosen for detailed analysis in that study (clone H99), exposure to the agonist epinephrine for 24 h led to an *up*-regulation of α_{1B}-adrenoceptors rather than the expected down-regulation. In further studies, we have now found that this up-regulation in clone H99 is an atypical response, and that most clones of α_{1B}-adrenoceptor-transfected CHO cells exhibit the expected *down*-regulation in response to agonist treatment. The possible role of protein kinases in the up-regulation observed in clone H99 has also been investigated, and additional interesting results have been obtained.

The primary signal transduction pathway for α_{1B}-adrenoceptors is thought to be phosphoinositide hydrolysis by phospholipase C, leading to inositol trisphosphate production and intracellular Ca^{2+} release and to diacylglycerol production and activation of protein kinase C (Graham et al., 1996). We hypothesized that protein kinase C activation might be involved in the agonist-induced up-regulation of α_{1B}-adrenoceptors in transfected CHO cells. Accordingly, the effects of staurosporine and other protein kinase inhibitors on α_{1B}-adrenoceptor expression in these

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cells were investigated. Our results do not support the involvement of protein kinase C in up-regulation. However, they are consistent with the involvement of other, perhaps multiple, protein kinases in this novel up-regulation phenomenon. They also suggest the existence of an unidentified cellular response element that can be activated by epinephrine and by staurosporine and inhibited by genistein.

2. Materials and methods

2.1. Materials

Fetal bovine serum, cell culture medium, trypsin and G418 were from GibcoBRL (Grand Island, NY). [^{3}H]Prazosin was from NEN Biotech Systems (Wilmington, DE) and [^{35}S]methionine was from ICN Radiochemicals (Irvine, CA). GF109203X was from Biomol Research Labs (Plymouth Meeting, PA) and genistein and daidzein were from Calbiochem (San Diego, CA). Staurosporine, epinephrine, phentolamine, phorbol 12-myristate, 13-acetate (PMA), cycloheximide and other biochemicals were from Sigma Chemical (St. Louis, MO).

2.2. α_{1B}-Adrenoceptor expression in CHO cells

The preparation and initial characterization of the CHO cells transfected with the hamster α_{1B}-adrenoceptor that were used in these studies have been described previously (Zhu et al., 1996). Briefly, the cDNA encoding the hamster α_{1B}-adrenoceptor (Cotecchia et al., 1988; obtained from R. Lefkowitz, Duke University, Durham, NC) was transferred into the plasmid pRC/CMV and transfected into CHO cells via calcium phosphate precipitation. G-418-resistant clones were isolated and screened for α_{1B}-adrenoceptor expression by [^{3}H]prazosin binding. Clone H99, which expressed approximately 230,000 receptors per cell, was chosen for further studies of receptor function and regulation and was the clone used in the studies reported previously (Zhu et al., 1996). Most of the experiments reported here also utilized clone H99, but clone H88 and several other clones from the same transfection and selection and additonal clones from a subsequent transfection and selection (Wang et al., 1997) were used in some of the studies reported here.

2.3. Cell culture and drug treatments

Cells were subcultured in 35-mm plastic culture dishes at 15–50,000 cells/dish as previously described (Zhu et al., 1996). On the third or fourth day of culture, cells were exposed to the indicated concentrations of protein kinase inhibitors in the absence or presence of epinephrine for the indicated times. The vehicle for epinephrine was ascorbate (1 mM final concentration) and for the kinase inhibitors was dimethyl sulfoxide (1% final concentration), and control cells were incubated with vehicle alone in all cases. For experiments with cycloheximide, cycloheximide was added approximately 4 h prior to addition of protein kinase inhibitor or vehicle.

2.4. α_{1B}-Adrenoceptor binding assays

[^{3}H]Prazosin binding assays were conducted on intact cells as in our previous study (Zhu et al., 1996). In most experiments, binding of a single high concentration of [^{3}H]prazosin (1.7 nM) was measured. Following the various pretreatments, cells on 35-mm culture dishes were washed and incubated for 60 min at 37°C in Ham's F12 medium buffered to pH 7.5 with 20 mM HEPES (Ham's-HEPES) containing [^{3}H]prazosin in the absence or presence of 10 μM phentolamine to define nonspecific binding. Cells were then washed two or three times with 37° Ham's-HEPES and dissolved in 1 ml of 0.2 N NaOH. Radioactivity was quantitated by liquid scintillation counting.

For saturation binding assays, the binding was essentially identical to that above, except that the concentration of [^{3}H]prazosin was varied. Before washing at the end of the binding reaction, an aliquot of the supernatant medium was taken for scintillation counting to determine the concentration of free radioligand. Bound radioligand associated with the cells was determined as described above.

2.5. Additional procedures

In some experiments, cells were counted with a hemocytometer following removal from the dishes with trypsin. Analysis of all binding data, including curve-fitting and statistical comparisons, was performed with GraphPad Prism (San Diego, CA).

3. Results

3.1. Regulation of α_{1B}-adrenoceptor expression by epinephrine in multiple clones of α_{1B}-adrenoceptor-transfected CHO cells

The finding of agonist-induced up-regulation of α_{1B}-adrenoceptors in our previous study (Zhu et al., 1996) was unexpected, since exposure of cells to agonist generally leads to down-regulation of receptor expression. As indicated in that study, the up-regulation observed in clone H99 did not appear to be an artefact of the high level of expression of α_{1B}-adrenoceptors in these cells, since up-regulation was also observed in preliminary experiments with another clone (L3) that expressed lower levels of α_{1B}-adrenoceptors. However, in experiments with clone H88, another relatively high-expressing clone from the same transfection and selection as the H99 cells,

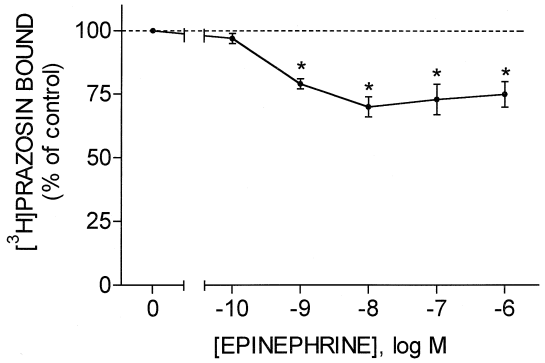

Fig. 1. Epinephrine-induced down-regulation of α_{1B}-adrenoceptor expression in clone H88. H88 cells were incubated for 24 h in the absence (0) or presence of the indicated concentrations of epinephrine. Cells were then washed and binding of [^{3}H]prazosin to intact cells was measured. Data are expressed as the percentage of binding to control cells incubated in the absence of epinephrine and are the means ± S.E.M. from five experiments, each performed in duplicate or triplicate. *, the value in the presence of epinephrine is significantly different from the control value; $P < 0.01$ by repeated measures ANOVA with Dunnett's multiple comparison post-test.

epinephrine was found to induce *down*-regulation of α_{1B}-adrenoceptors, decreasing [^{3}H]prazosin binding to intact cells (Fig. 1). The down-regulation in H88 cells occurred at very low concentrations of epinephrine, with half-maximal down-regulation at approximately 1 nM epinephrine, the same concentration that induced half-maximal up-regulation in H99 cells in our previous study (Zhu et al., 1996).

These results indicated that different clones from the same transfection can exhibit markedly different properties of long-term receptor regulation, so further studies were conducted with additional clones from this transfection and with multiple clones from a subsequent transfection (Wang et al., 1997). These studies indicate that epinephrine induces down-regulation under these conditions in most clones. Ten additional clones tested in further experiments all exhibited down-regulation in response to epinephrine, with an average down-regulation of 32 ± 3% ($n = 19$). However, both the H99 cells and the L3 cells reported to show up-regulation in our previous study (Zhu et al., 1996) again exhibited up-regulation in response to epinephrine in these experiments. Thus up-regulation is consistently observed in clones H99 and L3, but this is an atypical response not observed in most other clones. The molecular mechanisms involved in this novel up-regulation phenomenon were further investigated.

3.2. Effects of staurosporine on α_{1B}-adrenoceptor expression in H99 cells

To test for the possible involvement of protein kinase C activation in the agonist-induced up-regulation of α_{1B}-adrenoceptors reported previously for clone H99, the effects of the protein kinase C inhibitor staurosporine (Tamaoki et al., 1986) were investigated. Exposure of clone H99 to staurosporine for 24 h induced a concentration-dependent up-regulation of [^{3}H]prazosin binding, with maximal up-regulation of 2.0 ± 0.2-fold (Fig. 2A). The up-regulation occurred at low concentrations of staurosporine, with half-maximal up-regulation at approximately 1 nM staurosporine. Exposure to 10 nM epinephrine induced a similar extent of up-regulation (1.7 ± 0.2-fold), as in our previous studies (Zhu et al., 1996). In the

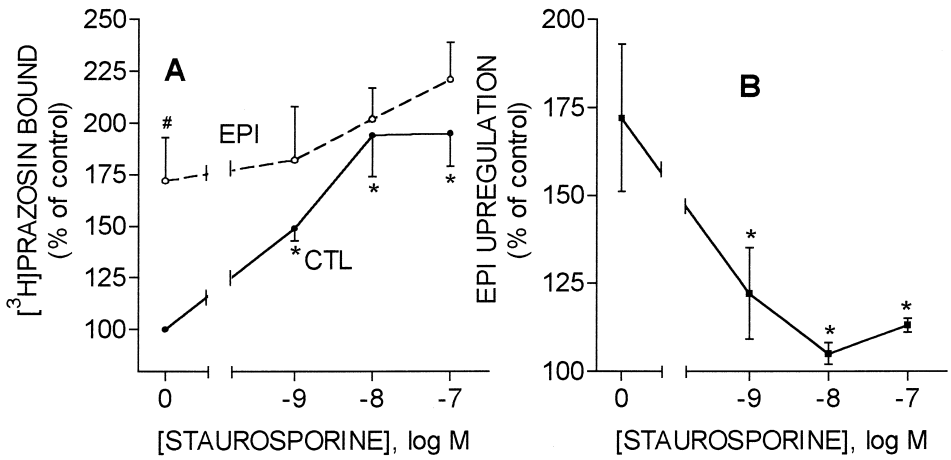

Fig. 2. Effects of staurosporine on α_{1B}-adrenoceptor expression and on epinephrine-induced up-regulation. *Panel A.* H99 cells were incubated for 24 h in the absence (0) or presence of the indicated concentrations of staurosporine in the absence (control, CTL, ●) or presence of 10 nM epinephrine (EPI, ○). Cells were then washed and binding of [^{3}H]prazosin to intact cells was measured. Data are expressed as the percentage of binding to control cells incubated in the absence of both staurosporine and epinephrine and are the means ± S.E.M. from three experiments, each performed in triplicate. *, the CTL value in the presence of staurosporine is significantly different from the value in the absence of staurosporine, $P < 0.01$ by repeated measures ANOVA with Newman-Keuls post-test. #, the value in the presence of epinephrine is significantly different from the CTL value for the same staurosporine concentration; $P < 0.05$ by repeated measures ANOVA with Newman-Keuls post-test. *Panel B.* Data from panel A are expressed as the percent up-regulation induced by epinephrine (EPI) in the presence of the indicated concentrations of staurosporine. *, the up-regulation induced by epinephrine in the presence of staurosporine is signicantly different from that in the absence of staurosporine, $P < 0.05$ by repeated measures ANOVA with Dunnett's multiple comparison post-test.

presence of epinephrine, staurosporine caused a modest further up-regulation (to 2.2 ± 0.2-fold above control), rather than inhibiting the epinephrine-induced up-regulation as originally expected. When the data are replotted as the fold up-regulation induced by epinephrine at different staurosporine concentrations (Fig. 2B), it appears that staurosporine markedly inhibited the epinephrine-induced up-regulation. However, this is clearly due to the fact that staurosporine caused up-regulation on its own, and epinephrine is apparently not able to induce significant further up-regulation beyond that induced by staurosporine.

To determine whether the increase in [^{3}H]prazosin binding induced by staurosporine was due to an increase in B_{max} or a decrease in K_D (or both), saturation binding assays were performed on control and staurosporine-treated cells (Fig. 3). The data from three experiments indicated an average 1.9 ± 0.2-fold increase in B_{max}, from 860 ± 70 fmol/dish for control cells to 1630 ± 130 fmol/dish for cells treated with 10 nM staurosporine ($P < 0.05$, paired t-test, $n = 3$). Similar to results with epinephrine (Zhu et al., 1996), staurosporine treatment appeared to decrease the attachment of cells to the dish, leading to an approximately 20% decrease in the number of cells remaining on the dish at the end of the binding assay in staurosporine-treated dishes compared to the control dishes in this set of experiments. When the binding data are expressed as receptors/cell to correct for this effect, the average staurosporine-induced up-regulation is 2.6 ± 0.8-fold, from $473{,}000 \pm 21{,}000$ receptors/cell in control cells to $1{,}197{,}000 \pm 310{,}000$ receptors/cell in staurosporine-treated cells. Treatment with staurosporine also caused a modest but consistent decrease in affinity for [^{3}H]prazosin, averaging 1.5 ± 0.1-fold, with K_D values of 260 ± 70 pM in control cells and 400 ± 110 pM in cells treated with staurosporine ($P < 0.1$, paired t-test, $n = 3$), similar to previous results for the up-regulation induced by epinephrine in these cells (Zhu et al., 1996). As reported previously for epinephrine-induced up-regulation, up-regulation was also observed in assays with membrane preparations isolated from staurosporine-pretreated cells (data not shown), indicating that the up-regulation is not an artefact of the intact cell binding assay routinely used in our experiments.

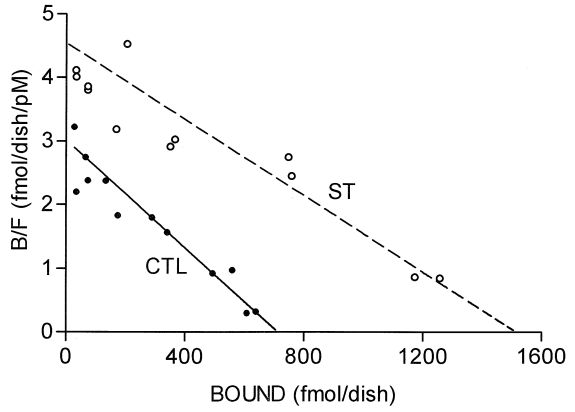

Fig. 3. Saturation binding analysis of [^{3}H]prazosin binding to control and staurosporine-treated cells. Binding of different concentrations of [^{3}H]prazosin to intact H99 cells pretreated for 24 h in the absence (control, CTL, ●) or presence of 10 nM staurosporine (ST, ○) was determined. Data were fit by computerized nonlinear regression analysis and are presented in the form of a Rosenthal plot (Rosenthal, 1967). The data shown are the duplicate data points from one of three similar experiments; average values and statistical analyses from multiple experiments are presented in the text.

3.3. Effects of GF109203X and PMA on α_{1B}-adrenoceptor expression

To further investigate the involvement of protein kinase C in these up-regulation phenomena, the effects of the more selective protein kinase C inhibitor GF109203X (Toullec et al., 1991) were assessed (data not shown). GF109203X did not mimic the staurosporine-induced up-regulation of α_{1B}-adrenoceptors at concentrations ranging from 10 nM to 10 μM. Furthermore, GF109203X inhibited epinephrine-induced up-regulation only at concentrations of 1 μM or greater, higher concentrations than the low nM concentrations at which inhibition of protein kinase C occurs (Toullec et al., 1991). These results with GF109203X suggest that neither the up-regulation by staurosporine nor that by epinephrine are likely to be mediated through protein kinase C activation.

The effects of the protein kinase C activator PMA were also assessed (data not shown). Exposure of cells to PMA at concentrations from 0.1 nM to 1 μM for either 6–7 h or for 24 h led to only minor changes in the binding of [^{3}H]prazosin. These results again suggest that neither the up-regulation by staurosporine nor that by epinephrine are likely to be mediated through protein kinase C activation.

3.4. Effects of genistein and daidzein on α_{1B}-adrenoceptor expression

The effects of the tyrosine kinase inhibitor genistein and its weaker analog daidzein (Akiyama et al., 1987) on α_{1B}-adrenoceptor expression in control cells and on epinephrine-induced up-regulation were assessed. Genistein did not induce up-regulation as staurosporine did, with little or no change in binding observed in cells treated with genistein at concentrations from 1 to 30 μg/ml; similar results were obtained in analagous experiments with the weaker genistein analog daidzein (data not shown). However, genistein did cause a concentration-dependent inhibition of the epinephrine-induced up-regulation, with half-maximal inhibition at approximately 5 μg/ml genistein (Fig. 4, top), a typical potency for genistein inhibition of tyrosine kinases (Akiyama et al., 1987). Similar inhibition was observed with daidzein (Fig. 4, top), although the

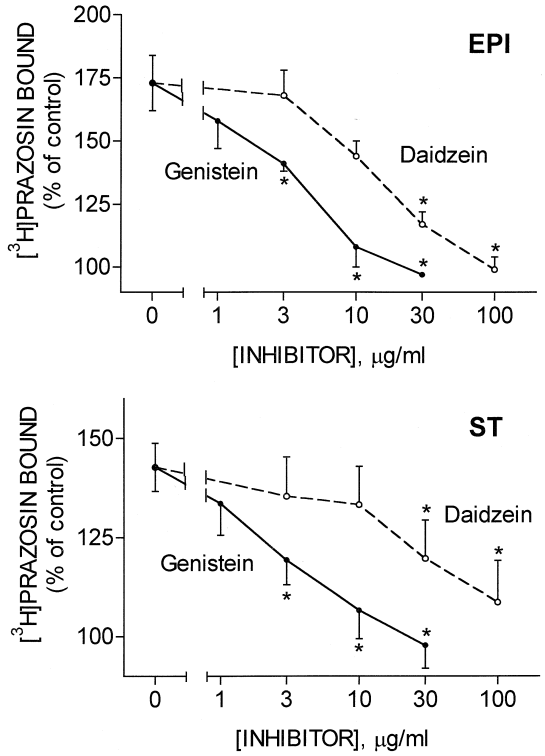

Fig. 4. Inhibition by genistein and daidzein of epinephrine- and staurosporine-induced up-regulation of α_{1B}-adrenoceptors. H99 cells were incubated for 24 h in the absence or presence of the indicated concentrations of genistein or daidzein in the absence (control) or presence of 10 nM epinephrine (EPI, top panel) or 10 nM staurosporine (ST, bottom panel). Cells were then washed and binding of [^{3}H]prazosin to intact cells was measured. Data are expressed as the percentage of binding to control cells incubated in the absence of genistein or daidzein and are the means ± S.E.M. from three experiments for epinephrine and five experiments for staurosporine, each performed in triplicate. *, the extent of up-regulation in the presence of inhibitor is significantly less than that in the absence of inhibitor, $P < 0.05$ by repeated measures ANOVA with Dunnett's multiple comparison post-test.

concentrations required were higher than with genistein, as expected (Akiyama et al., 1987).

The effects of genistein and daidzein on the up-regulation induced by staurosporine were also investigated (Fig. 4, bottom). Results nearly identical to those for epinephrine-induced up-regulation were obtained, with genistein causing half-maximal inhibition at approximately 5 μg/ml and daidzein exhibiting approximately 10-fold lower potency. These results suggest that staurosporine and epinephrine may induce up-regulation by similar mechanisms.

3.5. Effects of cycloheximide on epinephrine- and staurosporine-induced up-regulation

The effects of cycloheximide were assessed to determine whether the up-regulation induced by epinephrine and by staurosporine involved new protein synthesis. Cycloheximide markedly inhibited the up-regulation induced by epinephrine (Fig. 5, top), with 85% inhibition of up-regulation at 5 μg/ml cycloheximide, indicating that most of the up-regulation requires ongoing protein synthesis. However, protein synthesis assessed as [^{35}S]methionine incorporation into trichloroacetic acid-precipitable material was inhibited by greater than 99% under these same conditions, leaving open the possibility that a portion of the epinephrine-induced up-regulation is independent of protein synthesis.

In the case of staurosporine-induced up-regulation (Fig. 5, bottom), cycloheximide at 0.5 μg/ml was sufficient to completely eliminate up-regulation, and at higher concentrations of cycloheximide a staurosporine-induced decrease in [^{3}H]prazosin binding was revealed. These results suggest that staurosporine is inducing both a protein

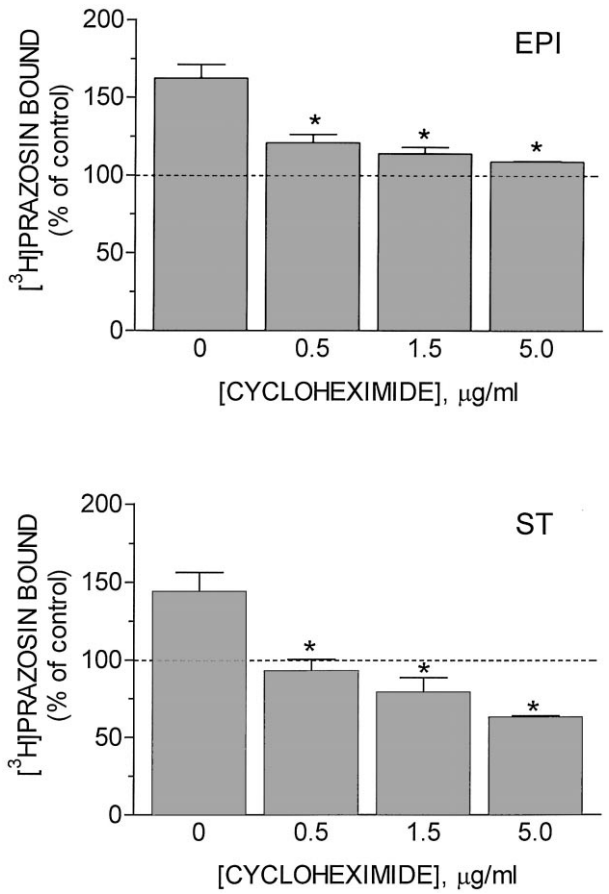

Fig. 5. Effects of cycloheximide on epinephrine- and staurosporine-induced up-regulation of α_{1B}-adrenoceptors. H99 cells were incubated in the absence or presence of 10 nM epinephrine (EPI, top panel) or 10 nM staurosporine (ST, bottom panel) in the absence (0) or presence of 0.5, 1.5, or 5.0 μg/ml of cycloheximide. Cells were then washed and binding of [^{3}H]prazosin to intact cells was measured. Data are expressed as the percentage of binding to control cells incubated with the same concentration of cycloheximide in the absence of epinephrine or staurosporine and are the means ± S.E.M. from three experiments, each performed in triplicate. *, the value in the presence of cycloheximide is significantly less than the value in the absence of cycloheximide; $P < 0.01$ by repeated measures ANOVA with Dunnett's multiple comparison post-test.

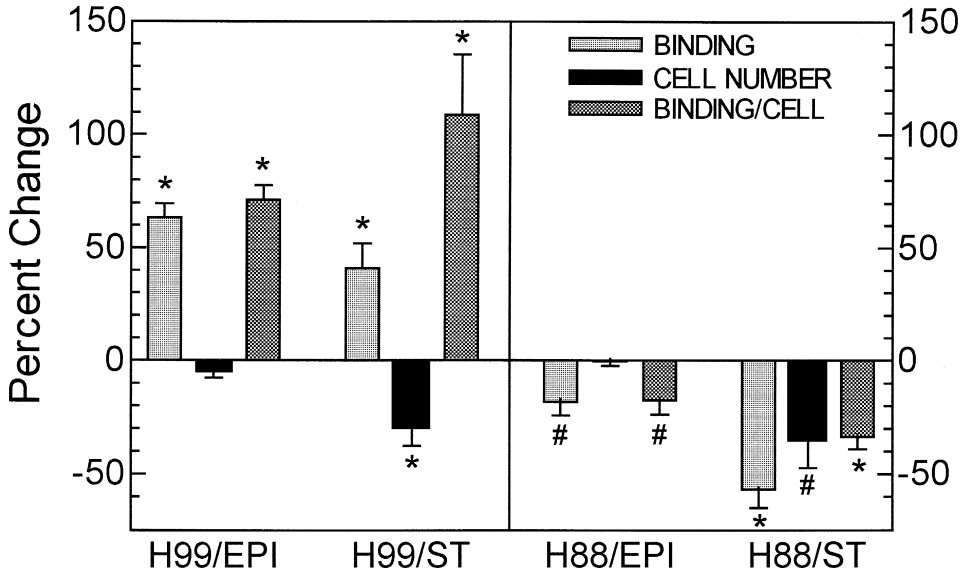

Fig. 6. Effects of epinephrine and staurosporine on [^{3}H]prazosin binding and cell attachment for clones H99 and H88. H99 and H88 cells were incubated for 24 h in the absence or presence of 10 nM epinephrine (EPI) or 10 nM staurosporine (ST). Cells were then washed and binding of [^{3}H]prazosin to intact cells and the number of cells remaining per dish were measured. Data are presented as the percent change induced by epinephrine and staurosporine and are the means ± S.E.M. from four or five experiments, each performed in triplicate. 'BINDING' represents [^{3}H]prazosin bound per dish, 'CELL NUMBER' represents the number of cells remaining per dish, and 'BINDING/CELL' represents the ratio of 'BINDING' to 'CELL NUMBER'. Statistical significance of the changes (assessed by a one-sample *t*-test for the difference of each value from 0) is indicated by * ($P < 0.05$) and # ($P < 0.10$).

synthesis-dependent up-regulation of α_{1B}-adrenoceptors and a protein synthesis-independent decrease in α_{1B}-adrenoceptors.

3.6. Comparison of effects of epinephrine and staurosporine on α_{1B}-adrenoceptor expression and cell attachment in clones H99 and H88

Because staurosporine was able to mimic the epinephrine-induced up-regulation in clone H99, we tested whether staurosporine might also mimic the epinephrine-induced down-regulation observed in clone H88 (Fig. 6). In this set of experiments, epinephrine induced approximately 60% up-regulation of [^{3}H]prazosin binding in H99 cells and approximately 20% down-regulation in H88 cells, similar to results from previous experiments. Epinephrine-induced detachment of cells was minimal in these experiments, and so the data expressed as binding/cell are similar to the raw binding data (Fig. 6). Staurosporine induced effects qualitatively similar to those with epinephrine in both clones, with approximately 40% up-regulation of [^{3}H]prazosin binding in H99 cells and nearly 60% apparent down-regulation in H88 cells (Fig. 6). However, the detachment of cells from the culture dishes treated with staurosporine was much greater than for those treated with epinephrine for both H99 and H88 cells, with an approximately 30% decrease of cell number compared to control dishes (Fig. 6). Accordingly, when expressed as number of binding sites per cell, the staurosporine-induced up-regulation in H99 cells was even greater, with an approximately 110% increase. On the other hand, some of the apparent staurosporine-induced down-regulation in H88 cells was in fact due to loss of cells, and the down-regulation when expressed as number of binding sites per cell was somewhat smaller, approximately 35% (Fig. 6).

4. Discussion

In clone H99 of CHO cells transfected with the α_{1B}-adrenoceptor, 24 h exposure to agonist induced up-regulation of receptors rather than the expected down-regulation (Zhu et al., 1996). The studies with additional clones presented here reveal that the up-regulation observed in clone H99 and in the lower-expressing clone L3 is atypical, and that 24 h agonist exposure leads to down-regulation in most clones. Although the response of H99 cells is atypical, investigation of potential mechanisms involved in this unexpected up-regulation suggest that a novel signaling pathway may be activated by α_{1B}-adrenoceptors in these cells.

Modulators of protein kinases were used to investigate the possible involvement of protein kinase C or other protein kinases in agonist-induced up-regulation. Protein kinase C seemed a likely candidate, since agonist binding to α_{1B}-adrenoceptors leads to activation of phosphoinositide hydrolysis and subsequent activation of protein kinase C (Graham et al., 1996). However, the protein kinase C activator PMA was unable to mimic agonist-induced up-regulation and the selective protein kinase C inhibitor

GF109203X (Toullec et al., 1991) was unable to block agonist-induced up-regulation at protein kinase C-selective concentrations, suggesting that protein kinase C activation is not the mechanism for this up-regulation. However, the ability of higher concentrations of GF109203X to block up-regulation is consistent with the possible involvement of a different protein kinase in epinephrine-induced up-regulation.

The tyrosine kinase inhibitor genistein did inhibit agonist-induced up-regulation, and the structurally similar but less potent analog daidzein inhibited up-regulation also but with lower potency. These results suggest the possible involvement of a tyrosine kinase pathway in mediating this agonist-induced up-regulation (Akiyama et al., 1987). Tyrosine kinase activation is not typically associated with activation of α_{1B}-adrenoceptors or other G-protein-coupled receptors. However, recent studies indicate that some tyrosine kinase pathways, particularly cytoplasmic tyrosine kinases, can be activated by G-protein $\beta\gamma$ subunits following activation of G-protein-coupled receptors (Post and Brown, 1991). Further studies will be required to confirm a role for tyrosine kinases in α_{1B}-adrenoceptor up-regulation and to identify the specific kinases involved.

Quite unexpectedly, the protein kinase inhibitor staurosporine, often used as a protein kinase C inhibitor (Tamaoki et al., 1986), induced up-regulation of α_{1B}-adrenoceptor binding on its own in clone H99. However, up-regulation was not induced by GF109203X, another protein kinase C inhibitor, or by the tyrosine kinase inhibitor genistein, suggesting that staurosporine may induce up-regulation by a novel mechanism. Up-regulation was staurosporine concentration-dependent and occurred at very low concentrations of staurosporine, with nearly maximal up-regulation with only 10 nM staurosporine. With staurosporine pretreatment, as in our previous studies with epinephrine pretreatment (Zhu et al., 1996), there was an increase of approximately 2-fold in the B_{max} and a modest increase in the K_D for [^{3}H]prazosin binding. These similarities raise the intriguing possibility that epinephrine and staurosporine may be acting on the same pathway to induce up-regulation by a common final mechanism. The fact that epinephrine and staurosporine together induced little more up-regulation than either agent alone is also consistent with this idea. Finally, genistein and daidzein blocked the up-regulation induced by staurosporine with essentially identical potencies as for their inhibition of epinephrine-induced up-regulation, further supporting the idea that epinephrine and staurosporine may induce up-regulation by the same mechanism. The details of the pathway by which the protein kinase inhibitor staurosporine can activate an apparently protein kinase-mediated up-regulation of α_{1B}-adrenoceptors in these cells is an intriguing area for further investigation.

In addition to inducing up-regulation, exposure of H99 cells for 24 h to either epinephrine or staurosporine also caused some loss of cells from the culture dishes during the multiple washes involved in the binding procedure, as compared to control cells. This effect was typically more pronounced for staurosporine than for epinephrine. Both the decrease in α_{1B}-adrenoceptor affinity for [^{3}H]prazosin and the loss of cells that occurs with epinephrine or staurosporine treatment cause the magnitude of up-regulation to be somewhat underestimated in most of the experiments presented here. The saturation binding experiments in Fig. 3 provide the most accurate assessment of the magnitude of up-regulation, since saturation assays were performed and cells were counted to compensate for both the decrease in binding affinity and the loss of cells. Staurosporine is known to induce apoptosis (Bertrand et al., 1994), in which cells may become rounded and detach from the dish. Whether the loss of cells from the dish with staurosporine treatment under our conditions is due to apoptosis, and whether α_{1B}-adrenoceptor activation with epinephrine can also induce apoptosis are interesting questions for further investigation.

Cycloheximide markedly inhibited the up-regulation induced by epinephrine and by staurosporine, indicating a requirement for ongoing protein synthesis for up-regulation to occur. In the case of staurosporine, cycloheximide not only inhibited up-regulation, but also revealed an apparent staurosporine-induced down-regulation. The magnitude of this apparent down-regulation is similar to the magnitude of cell loss quantitated in other experiments. Thus we propose that staurosporine induces two distinct effects, a protein synthesis-dependent up-regulation of the number of α_{1B}-adrenoceptors that is superimposed on a protein synthesis-independent loss of cells from the dish.

Several other recent studies have observed agonist-induced up-regulation of various G-protein-coupled receptors, including dopamine receptors (Ivins et al., 1991; Filtz et al., 1993; Zhang et al., 1994; Starr et al., 1995), serotonin receptors (Akiyoshi et al., 1993) and β_3-adrenoceptors (Thomas et al., 1992). In some cases these phenomena occurred in cells normally expressing these receptors (Ivins et al., 1991; Thomas et al., 1992; Akiyoshi et al., 1993), whereas other cases involved transfected cell systems (Filtz et al., 1993; Zhang et al., 1994; Starr et al., 1995). Surprisingly, in some of the studies with dopamine receptors, both agonists and antagonists led to receptor up-regulation (Filtz et al., 1993; Starr et al., 1995). To our knowledge, up-regulation of G-protein-coupled receptors by staurosporine or other protein kinase inhibitors has not been reported previously. However, staurosporine has been reported to induce up-regulation of serotonin transporters in human choriocarcinoma cells (Ramamoorthy et al., 1995). Cyclic AMP-dependent mechanisms have been proposed to be involved in some cases of agonist-induced receptor up-regulation (Thomas et al., 1992; Filtz et al., 1993) but not in others (Zhang et al., 1994). In fact, elevation of cyclic AMP has been reported to cause up-regulation of α_{1B}-adrenoceptors or their mRNA in DDT_1 MF-2 cells (Morris et al., 1991; Schachter and Wolfe,

1995). Although these receptors are not classically thought to be coupled to cyclic AMP signalling, transfected α_{1B}-adrenoceptors have been shown to couple to stimulation of cyclic AMP production (Horie et al., 1995) and we have observed similar results with our transfected cells (data not shown). The possible role of cyclic AMP-dependent pathways in both the epinephrine- and staurosporine-induced up-regulation of α_{1B}-adrenoceptors in H99 cells remains to be established.

Finally, it should be emphasized that our studies were conducted with transfected cells that do not normally express α_{1B}-adrenoceptors and that the observed up-regulation by epinephrine and by staurosporine may not reflect processes that normally regulate α_{1B}-adrenoceptor expression in cells that express these receptors endogenously. This is underscored by the fact that clone H88 and most other clones were found to undergo down-regulation of α_{1B}-adrenoceptors in response to epinephrine. Down-regulation rather than up-regulation was also observed with staurosporine in these other clones, although the extent to which cell detachment contributes to this down-regulation was studied only for clone H88. Our results emphasize that considerable caution should be used in interpreting studies of receptor regulation in transfected cells. This is particularly true for studies of longer-term regulation, such as down-regulation, where changes in gene expression are likely to be involved. In such cases, the regulatory properties observed may be highly dependent on the specific plasmid used, the amount of upstream and downstream regulatory sequence from the plasmid that becomes incorporated into the recipient cell genome, and the specific sites at which the plasmid becomes incorporated. Such differences probably account for the different regulatory responses to epinephrine and staurosporine in clone H99 versus clone H88 and most other clones, although this remains to be established. On the other hand, it should also be stressed that studies of unexpected regulatory phenomena that are observed in such transfected cell systems, such as the up-regulation studied here, have the potential to reveal new receptor signalling pathways and/or new genetic regulatory mechanisms that may be involved in controlling expression of various endogenous genes as well. Thus, further studies of the molecular pathway by which epinephrine and staurosporine up-regulate α_{1B}-adrenoceptor expression in clone H99 may be informative, even though this response is atypical. For example, staurosporine has recently been reported to mediate several cellular effects independent of its effects on protein kinase C (Fallon, 1990; Rasouly et al., 1992; Sowa and Przewlocki, 1995), and staurosporine-induced up-regulation of α_{1B}-adrenoceptors in clone H99 could provide a convenient system for investigating these additional mechanisms of action for staurosporine. Our studies suggest the possible existence of a novel response element that can be activated by epinephrine and by staurosporine and inhibited by genistein. Identification of such a response element and the specific genes or cellular responses which it normally regulates could prove interesting.

In summary, both epinephrine and staurosporine induce up-regulation of α_{1B}-adrenoceptors in an atypical clone of transfected CHO cells, but both induce down-regulation in most other clones. Protein kinase C activation does not appear to be the mechanism for up-regulation, but tyrosine kinases or other protein kinases regulating a protein synthesis-dependent pathway are likely to be inovlved. The unexpected results presented here underscore the fact that studies in transfected cell systems can reveal useful information on regulation of receptor expression but that studies in these systems should be interpreted with considerable caution.

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