ABSTRACT

We examined the down-regulation of α1B-adrenoceptors in Madin-Darby canine kidney-derived (MDCK) cells with an emphasis on a possible role of protein kinase C (PKC). The α1-adrenoceptor agonist phenylephrine (1–100 μM) concentration-dependently down-regulated α1B-adrenoceptors in MDCK cells. Down-regulation by 100 μM phenylephrine was detectable after 2 hr and maximal after 8 to 24 hr. The receptor down-regulation was accompanied by a decrease in phenylephrine-stimulated inositol phosphate formation but not by an altered expression of immunodetectable Gq/11 α subunits. Even though α1B-adrenoceptor and PGE2 purinergic receptor stimulation promote prostaglandin E2 formation, receptor down-regulation was not prevented by indomethacin (10 μM) treatment but was partly mimicked by treatment with the purinergic receptor agonists adenosine-5′-O-(3-thio)triphosphate and 2-methylthio-ATP (300 μM each). Phorbol-12-myristate-13-acetate (1–100 nM) concentration-dependently down-regulated MDCK α1B-adrenoceptors to a greater extent than did phenylephrine. Three protein kinase C inhibitors, H7 (100 μM), staurosporine (100 nM) and KT5926 (1 μM), markedly attenuated receptor down-regulation promoted by phorbol ester but did not affect that by phenylephrine. Two inhibitors of Ca2+/calmodulin protein kinase pathways, KT5926 (1 μM) and W-7 (30 μM), also failed to prevent phenylephrine-induced down-regulation of α1B-adrenoceptors. We conclude that agonist-induced down-regulation of MDCK cell α1B-adrenoceptors is mimicked by a protein kinase C-activating phorbol ester but that the second messenger kinases protein kinase C and Ca2+/calmodulin protein kinase do not mediate agonist-induced down-regulation of the α1B-adrenoceptor.

Desensitization, a process by which cells adapt to extended exposure to agonists, results in a diminished functional response despite continued presence of the agonist and/or the requirement for a greater agonist concentration to maintain a given response level. Among the G protein-coupled receptor desensitization has been most extensively investigated for the β2-adrenoceptor (Hausdorff et al., 1990; Hadcock and Malbon, 1993; Barnes, 1995; Premont et al., 1995). Thus, desensitization of the β2-adrenoceptor involves phosphorylation of the receptor, uncoupling from its G protein, sequestration into an intracellular compartment where it can no longer couple to the G protein and, finally, down-regulation of the receptor and/or components of its signaling machinery (e.g., G proteins). Agonist-promoted desensitization of β2-adrenoceptors involves two types of protein kinases, GRK and the second messenger kinase, cAMP-dependent protein kinase. Much less is known regarding the mechanisms of desensitization of other receptors, particularly those that activate other signaling mechanisms such as the phospholipase C/Ca2+/PKC pathway (e.g., the α1-adrenoceptors).

α1-adrenoceptors typically act by activating phosphatidylinositol phosphate hydrolysis to form inositol-1,4,5-triphosphate and diacylglycerol (Summers and McMartin, 1993). This results in activation of PKC. Similar to β2-adrenoceptors, desensitization of α1-adrenoceptors involves rapid processes such as sequestration and slower processes such as receptor down-regulation (Cotecchia et al., 1995; Garcia-Sainz, 1993). In recent years, some progress has been made in elucidating the mechanisms underlying rapid α1-adrenoceptor desensitization by sequestration, and it has become clear that this phase of desensitization occurs independent of PKC activation (Lattion et al., 1994). On the other hand, slower forms of desensitization may be more relevant for pathophysiological desensitization that may oc-

ABBREVIATIONS: ATPγS, adenosine-5′-O-(3-thio)triphosphate; GRK, G protein-coupled receptor kinases; MDCK, Madin-Darby canine kidney; PMA, phorbol-12-myristate-13-acetate; HEK, human embryonic kidney; PKC, protein kinase C.
cur in disease states associated with chronically elevated sympathoadrenal tone such as congestive heart failure (Packer, 1992) or chronic renal failure (Daul et al., 1987).

However, little is known regarding the mechanisms underlying alpha-1 adrenoceptor down-regulation in response to agonists.

MDCK cells are a cell line derived from distal nephron segments. They contain alpha-1 adrenoceptors, which couple to a pertussis toxin-insensitive G protein, possibly Go, to induce stimulation of phospholipase C, which results in activation of PKC and cytosolic phospholipase A2 (Godson et al., 1990; Slivka et al., 1988; Terman et al., 1987; Xing and Insel, 1996). Some data indicate that the MDCK cell alpha-1 adrenoceptors may belong to the alpha-1B subtype (Blue et al., 1994). We have previously demonstrated agonist-promoted desensitization and down-regulation of alpha-1B adrenoceptors in MDCK cells (Meier et al., 1985). In the present study, we used MDCK cells to further investigate agonist-induced desensitization and down-regulation of the alpha-1B adrenoceptor and, in particular, a possible role of PKC in these events.

**Methods**

**Cell culture.** MDCK cells were grown in an atmosphere of 5% CO2/95% air at 37° in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin and, unless otherwise indicated, 7.5% heat-inactivated horse serum and 2.5% heat-inactivated fetal calf serum. Subconfluent cells were subcultured every 3 to 4 days with a 0.5% gelatin trypsin and 0.2 g/liter Na2EDTA solution. In some experiments, cells were treated for the indicated times and with the indicated concentrations of the alpha-1 adrenoceptor agonist phenylephrine and/or various activators and/or inhibitors of PKC. All treatment experiments involving phenylephrine were performed in the presence of 10 µM (+)-propranolol to prevent concomitant beta adrenoceptor activation.

**Radioligand binding.** Confluent cells were harvested, counted, resuspended into ice-cold binding buffer (50 mM Tris, 0.5 mM EDTA, pH 7.4) and homogenized by an Ultra-Turrax (Janke and Kunkel, Staufen, Germany) for 10 sec at full speed and thereafter twice for 20 sec at each two-thirds speed. The homogenate was centrifuged for 20 min at 50,000 × g. Alpha-1B adrenoceptor affinity and number were determined by saturation binding of [3H]prazosin as previously described (Michel et al., 1993). Briefly, aliquots of the membrane suspension were incubated with six concentrations of [3H]prazosin (10–300 pM) in a total volume of 1000 µl for 45 min at 25°. The incubations were terminated by rapid vacuum filtration of Whatman GF/C filters. Nonspecific binding was defined as binding in the presence of 10 µM (+)-propranolol to prevent concomitant beta adrenoceptor activation.

**Radioligand binding saturation experiments were performed by analyzing rectangular hyperbolic functions to the experimental data using the InPlot program (GraphPAD Software, San Diego, CA). Competition binding experiments were analyzed by fitting monophasic and biphasic sigmoidal functions to the experimental data; a biphasic fit was accepted only if it resulted in a significant improvement as judged by an F test. Because some of our treatments altered cell number and/or cell size, we determined the number of cells and the total protein content per flask in each experiment. Radioligand binding and inositol phosphate formation data were analyzed by fitting rectangular hyperbolic functions to the experimental data using the InPlot program (GraphPAD Software, San Diego, CA).**
indicated that the variance between groups was significantly greater than that within groups, individual groups were compared with their respective control by Dunnet’s multiple comparison test. All statistical calculations were performed using the InStat program, and a value of P < .05 was considered significant.

Results

In untreated MDCK cells, [3H]prazosin bound to an apparently single class of sites with an affinity of 54 ± 3 pM and a density of 160 ± 7 fmol/mg protein, which was equivalent to 13,518 ± 627 sites/cell (n = 34). Competition binding experiments were performed with a panel nine subtype-selective agonists with a threshold concentration of 1 μM; a reduction in receptor density estimates in the radioligand binding experiments were not affected in a relevant manner by phenylephrine, which might have been retained from the pretreatments. On the other hand, some of the treatments affected MDCK cell number or size (assessed as protein content/cell); to avoid misinterpretation of the data due to such effects, we present our data in parallel using both protein content and cell number as denominators. Unless otherwise indicated, all further data on receptor number are expressed as percent of the receptor density measured in paired control cells.

Phenylephrine treatment (0.01–100 μM for 24 hr) in the presence of 10% serum did not significantly affect cell number or size, assessed as protein content per cell number (table 2). Under these conditions, phenylephrine treatment concentration-dependently reduced the number of α1B-adrenoceptors with a threshold concentration of 1 μM; a reduction of 49% was achieved by 100 μM phenylephrine. At all agonist concentrations, the extent of the down-regulation was similar when analyzed relative to protein content or cell number (fig. 1). In contrast, phenylephrine concentration-dependently increased cell number (control, 44 ± 3 × 10^6 cells/175-cm² flask; n = 4) by 13 ± 2%, 24 ± 3% and 29 ± 4% at 1, 10 and 100 μM phenylephrine, respectively (P < .05 vs. control for 10 and 100 μM phenylephrine) in the presence of 0.5% serum. This was accompanied by a trend for reduced cell size (control, 142 ± 13 μg of protein/10^6 cells) but failed to reach statistical significance with the given number of experiments. Under these conditions, phenylephrine also caused a concentration-dependent down-regulation of α1B-adrenoceptors (fig. 1). However, in 0.5% serum-containing medium, the extent of down-regulation was somewhat smaller and the threshold concentration for phenylephrine was higher than in standard (10%) medium. Moreover, in 0.5% serum-containing medium, the extent of down-regulation was somewhat greater when data were analyzed relative to cell number (40%) compared with relative to protein content (27%). Therefore, all further regulation studies were conducted in medium containing 10% serum. In time course experiments with 100 μM phenylephrine, statistically significant down-regulation of α1B-adrenoceptors was detected after 2 hr, and down-regulation was maximal with 8 to 24 hr of agonist treatment (fig. 2).

Incubation of the cells for 24 hr with the PKC activator PMA (1–100 nM) reduced cell number but did not significantly affect the size of the remaining cells (table 2). PMA (1–100 nM) concentration-dependently down-regulated MDCK cell α1B-adrenoceptors with maximal reductions exceeding those achieved by phenylephrine (fig. 3). The concentration-response curve for PMA was much steeper than that for phenylephrine (i.e., no significant effects were detected with 1 nM PMA but a 70–80% down-regulation occurred with 10 nM PMA).

Because the PKC activator PMA mimicked the effects of phenylephrine, we investigated whether the PKC inhibitors H7, staurosporine and KT5926 could block them. These experiments were limited by toxic effects of the PKC inhibitors. Thus, in the presence of 100 μM H7, 100 nM staurosporine or 1 μM KT5926 the cell number was reduced and the remaining cells appeared to be larger (table 2). Accordingly, in cells treated with H7, staurosporine or KT5926 basal α1B-adrenoceptor density was reduced to 45 ± 7%, 48 ± 2% and 83 ± 3%, respectively, of values in control cells (n = 4 each; P < .01). The presence of H7 (fig. 4), staurosporine (fig. 5) or KT5926 (fig. 6) markedly attenuated the 100 nM PMA-induced down-regulation of MDCK cell α1-adrenoceptors. On the other hand, neither H7 nor staurosporine significantly altered phenylephrine-induced down-regulation regardless of whether data were expressed relative to cell number or protein content (figs. 4 and 5). KT5926, which also can inhibit Ca^2+ /calmodulin-dependent protein kinase, enhanced phenylephrine-promoted down-regulation if data were expressed as sites/cells (P < .05) but not if expressed as fmol/mg protein (fig. 6). The calmodulin antagonist W-7 (30 μM) did not significantly affect cell number and size (table 2). Treatment with W-7 did not significantly affect the α1B-adrenoceptor down-regulation by PMA or phenylephrine (fig. 7).

The addition of the cyclooxygenase inhibitor indomethacin (10 μM) to the culture medium did not change the density of MDCK cell α1B-adrenoceptors or the ability of phenylephrine to down-regulate them (fig. 8). In contrast, a combination of the P2Y1 (P2Y1) purinergic receptor agonist 2-methylthio-ATP (300 μM) and the P2Y2 (P2Y2) purinergic receptor agonist ATPγS (300 μM) caused a small but statistically significant reduction of α1B-adrenoceptor expression, regardless whether data were expressed based on protein content or on cell number (fig. 8). The combined treatment with phenylephrine and the purinergic agonists did not cause additive down-regulation (fig. 8).

### TABLE 1

**Drug affinities at MDCK cell α1-adrenoceptors**

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Hill slope</th>
<th>− log Kᵢ</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Methylurapidil</td>
<td>1.04 ± 0.02</td>
<td>7.37 ± 0.14</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>0.83 ± 0.03</td>
<td>6.38 ± 0.07</td>
</tr>
<tr>
<td>Methoxamine</td>
<td>0.99 ± 0.04</td>
<td>4.34 ± 0.21</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>0.91 ± 0.00</td>
<td>6.79 ± 0.28</td>
</tr>
<tr>
<td>Oxymetazoline</td>
<td>1.06 ± 0.03</td>
<td>6.77 ± 0.04</td>
</tr>
<tr>
<td>Phenolamine</td>
<td>1.35 ± 0.27</td>
<td>8.27 ± 0.04</td>
</tr>
<tr>
<td>SDZ-NVI-085</td>
<td>1.16 ± 0.06</td>
<td>5.16 ± 0.03</td>
</tr>
<tr>
<td>(−)-Tamsulosin</td>
<td>0.98 ± 0.02</td>
<td>7.98 ± 0.03</td>
</tr>
<tr>
<td>(+)-Tamsulosin</td>
<td>1.04 ± 0.06</td>
<td>7.82 ± 0.06</td>
</tr>
</tbody>
</table>

Data are mean ± SEM of 3 or 4 experiments.
Effects of treatments on MDCK cell number and size

Effects of the indicated concentrations of pharmacological agents were tested during a 24-hr treatment of the cells in 10% serum-containing medium compared with cells treated with the respective vehicle in parallel flasks.

**TABLE 2**

Effects of treatments on MDCK cell number and size

<table>
<thead>
<tr>
<th>Cell number</th>
<th>Treated</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cells/175-cm² flask × 10⁻⁶</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylephrine (100 µM, n = 30)</td>
<td>36 ± 3</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>PMA (100 nM, n = 21)</td>
<td>39 ± 3</td>
<td>30 ± 2³</td>
</tr>
<tr>
<td>H7 (100 µM, n = 4)</td>
<td>67 ± 10</td>
<td>19 ± 3⁵</td>
</tr>
<tr>
<td>Staurosporine (100 nM, n = 4)</td>
<td>44 ± 4</td>
<td>12 ± 2⁵</td>
</tr>
<tr>
<td>KT5926 (1 µM, n = 4)</td>
<td>28 ± 1</td>
<td>11 ± 1⁵</td>
</tr>
<tr>
<td>W-7 (30 µM, n = 4)</td>
<td>37 ± 4</td>
<td>34 ± 3</td>
</tr>
</tbody>
</table>

σ, β, γ, δ P < .05, .01 and .0001, respectively, in a paired, two-tailed t test.

**Fig. 1.** Down-regulation of MDCK cell alpha-1 adrenoceptors by phenylephrine treatment. Cells were treated for 24 hr in the absence (control) and presence of the indicated phenylephrine concentrations in the presence of 10% (top) or 0.5% serum (bottom). Thereafter, receptor number was determined by [³H]prazosin saturation binding experiments. Receptor numbers were calculated as fmol/mg protein (■) or sites/cell (○), and are expressed as mean ± SEM of percentage of control values of 4 experiments. * and **, P < .05 and < .01, respectively, vs. control in a repeated-measures one-way analysis of variance followed by Dunnett’s multiple-comparison test.

In the immunoblot experiment, the QL antiserum, which is directed against a carboxyl-terminal sequence of G₁₅ and G₁₁, recognized a band with an apparent molecular mass of ∼40 kDa. The amount of [¹²⁵I]protein A bound to this band was not significantly affected by a 24-hr pretreatment of MDCK cells with 100 µM phenylephrine or 100 nM PMA (fig. 9).

Finally, we studied the effects of a 24-hr treatment of MDCK cells with vehicle, 100 µM phenylephrine or 100 nM PMA on the subsequent ability of phenylephrine to stimulate inositol phosphate formation. After vigorous washout of the pretreatments, cells were exposed for 45 min to 100 µM phenylephrine or vehicle (basal). Phenylephrine-induced inositol phosphate formation, expressed as percentage over basal values, was significantly reduced in phenylephrine-pretreated cells and even more so in PMA-pretreated cells when compared with control conditions (fig. 10).

**Discussion**

We used MDCK cells to study the agonist-induced down-regulation of alpha-1 adrenoceptors. A panel of nine subtype-selective agonists and antagonists had steep and monophasic competition curves indicating the presence of a homogeneous receptor population. The absolute drug affinities were consistent with those we previously reported using similar methods for alpha-1B adrenoceptors in rat spleen (Büscher et al., 1994; Michel et al., 1993) and rat liver (Büscher et al., 1994; Büscher et al., 1996) and with cloned rat alpha-1B adrenoeceptors (Büscher et al., 1994; Michel and Insel, 1994). Thus,
these and previous data (Blue et al., 1994) unequivocally demonstrate that MDCK cells express a homogeneous population of alpha-1B adrenoceptors. The down-regulation of the MDCK cell alpha-1B adrenoceptor was concentration- and time-dependent requiring 8 to 24 hr and 10 to 100 \( \mu \text{M} \) phenylephrine for maximum effects (i.e., a ~50% down-regulation). This could be observed under conditions where the alpha-1B adrenoceptor stimulation enhances MDCK cell proliferation (0.5% serum) and under conditions where it does not (10% serum). Thus, agonist-induced down-regulation is not dependent on cellular quiescence.

**Fig. 4.** Effects of H7 on down-regulation of alpha-1 adrenoceptors induced by phenylephrine (PE) or PMA. Cells were incubated with vehicle, 100 \( \mu \text{M} \) phenylephrine or 100 nM PMA in the absence (control; open bars) or presence of 100 nM H7 (hatched bars) for 24 hr. The receptor number was determined by \([^3\text{H}]\text{prazosin saturation binding experiments. Receptor numbers were calculated as fmol/mg protein (top) and sites/cell (bottom) and are expressed as mean \pm SEM of percentage of control values of 4 experiments. ***}, P < .001 vs. vehicle in a paired two-tailed \( t \) test.

**Fig. 5.** Effects of staurosporine on down-regulation of alpha-1 adrenoceptors induced by alpha-1 agonist phenylephrine (PE) or PMA. Cells were incubated with vehicle, 100 \( \mu \text{M} \) phenylephrine or 100 nM PMA in the absence (control; open bars) or presence of 100 nM staurosporine (hatched bars) for 24 hr. The receptor number was determined by \([^3\text{H}]\text{prazosin saturation binding experiments. Receptor numbers were calculated as fmol/mg protein (top) and sites/cell (bottom) and are expressed as mean \pm SEM of percentage of control values of 4 experiments. ** and ***}, P < .01 and < .001, respectively, vs. vehicle in a paired two-tailed \( t \) test.

**Fig. 6.** Effects of KT 4926 on down-regulation of alpha-1 adrenoceptors induced by alpha-1 agonist phenylephrine (PE) or PMA. Cells were incubated with vehicle, 100 \( \mu \text{M} \) phenylephrine or 100 nM PMA in the absence (control; open bars) or presence of 1 \( \mu \text{M} \) KT5926 (hatched bars) for 24 hr. The receptor number was determined by \([^3\text{H}]\text{prazosin saturation binding experiments. Receptor numbers were calculated as fmol/mg protein (top) and sites/cell (bottom) and are expressed as mean \pm SEM of percentage of control values of 4 experiments. * and ***}, P < .05 and < .001, respectively, vs. vehicle in a paired two-tailed \( t \) test.

**Fig. 7.** Effects of W-7 on down-regulation of alpha-1 adrenoceptors induced by alpha-1 agonist phenylephrine (PE) or PMA. Cells were incubated with vehicle, 100 \( \mu \text{M} \) phenylephrine or 100 nM PMA in the absence (control; open bars) or presence of 30 \( \mu \text{M} \) W-7 (hatched bars) for 24 hr. The receptor number was determined by \([^3\text{H}]\text{prazosin saturation binding experiments. Receptor numbers were calculated as fmol/mg protein (top) and sites/cell (bottom) and are expressed as mean \pm SEM of percentage of control values of 4 experiments. Differences in down-regulation by either agonist in the absence and presence of W-7 were not statistically significant in a paired, two-tailed \( t \) test.
endogenously expressed in cultured rat vascular smooth muscle cells (Kai et al., 1996). Studies on beta-2 adrenoceptor-induced down-regulation of Gα subunits have demonstrated that the loss of Gα may quantitatively depend on the expression density of the receptor coupled to this G protein (Adie and Milligan, 1994). This may explain the divergent results between the present study with natively expressed receptor and those with high densities of transfected receptors. Additionally, alterations of Gα expression on extended agonist exposure may depend on cell type under investigation and/or the degree of effectiveness with which the receptor couples to its effector mechanisms.

MDCK cell alpha-1 adrenoceptors not only couple to phospholipase C activation but also can activate phospholipase A2 to enhance arachidonic acid release (Xing and Insel, 1996). This results in the formation of prostaglandins, which might then act on prostanooid receptors to cause heterologous down-regulation of alpha-1B adrenoceptors. To investigate this possibility, experiments were performed in the presence of the cyclooxygenase inhibitor indomethacin. Because indomethacin did not affect the density of MDCK cell alphaB-adrenoceptor down-regulation or the ability of phenylephrine to cause their down-regulation, formation of cyclooxygenase-derived arachidonic acid metabolites does not appear to contribute to agonist-induced alphaB-adrenoceptor down-regulation. This hypothesis is further supported by our previous observation that alphaB-adrenoceptor-stimulated arachidonic acid release is PKC mediated (Xing and Insel, 1996), whereas phenylephrine-induced alphaB-adrenoceptor down-regulation is not (present study).

The possibility that PKC may be involved in alphaB-adrenoceptor down-regulation was indicated by several pieces of evidence. First, stimulation of MDCK alphaB-
adrenoceptors can activate PKC (Godson et al., 1990). Second, purinergic agonists, which also activate the phospholipase C/PKC cascade in MDCK cells (FIRESTEIN et al., 1996; YANG et al., 1997), at least partly mimicked alpha-1B adrenoceptor down-regulation. Third, and most important, several previous studies have demonstrated that PKC-activating phorbol esters can desensitize alpha-1B adrenoceptors. Short-term (10−30 min) incubation with PMA can uncouple the receptors from inositol phosphate formation in DDT1 hamster smooth muscle cells (Leeb-Lundberg et al., 1985). In rat liver, short-term PMA treatment can inhibit formation of the agonist high affinity state of the alpha-1B adrenoceptor (Beeler and Cooper, 1993; CORVERA et al., 1986). This is accompanied by a rapid, staurosporine-sensitive reduction of cell surface alpha-1B adrenoceptors and may involve receptor sequestration to intracellular compartments (Beeler and Cooper, 1995). Phorbol ester-induced alpha-1B adrenoceptor sequestration has also been observed in stably transfected HEK 293 cells (FONSECA et al., 1995). Some of these effects may result directly from receptor phosphorylation because PKC activation can cause alpha-1B adrenoceptor phosphorylation (BOUVIER et al., 1987). Down-regulation of alpha-1B adrenoceptors promoted by longer activation of PKC may involve other mechanisms. For example, in rabbit vascular smooth muscle cells, it may partly result from phorbol ester-induced destabilization of alpha-1 adrenoceptor mRNA (IZZO et al., 1994). On the other hand, PKC activation in hamster DDT, MF-2 cells can increase both mRNA accumulation and the number of alpha-1B receptors after 1-hr treatment, and these phenomena can continue up to 24 hr (HU et al., 1993).

In the present study, the PKC activator PMA concentration-dependently down-regulated MDCK cell alpha-1B adrenoceptors. This occurred with a very steep concentration-response relationship, and maximum reductions in receptor number exceeded those produced by the receptor agonist phenylephrine. PMA-promoted down-regulation was accompanied by functional desensitization, which quantitatively matched the down-regulation and was not accompanied by alterations in immunodetectable G$_{q,11}$. The alpha-1B adrenoceptor down-regulation by PMA was indeed PKC mediated because three distinct PKC inhibitors, H-7, staurosporine and KT5926, markedly inhibited it. Although down-regulation was not fully prevented with the applied inhibitor concentrations, higher concentrations could not be tested due to toxic effects of the compounds. It is intriguing that PMA treatment down-regulates MDCK cell alpha-1B adrenoceptors via PKC, although it concomitantly down-regulates several PKC isoforms in MDCK cells (GODSON et al., 1990). This could be explained if alpha-1B adrenoceptor down-regulation by PMA is a very rapid event preceding down-regulation of PKC and/or if it involves PKC isoforms that are resistant to down-regulation on PMA treatment.

Despite these effects of the antagonists on PMA-promoted down regulation of alpha-1B adrenoceptors, none of these three kinase inhibitors prevented phenylephrine-induced down-regulation. Although none of these inhibitors is very specific for PKC, all three were used in concentrations in which they effectively inhibit PKC. Although we cannot exclude that the effects of the PKC inhibitors alone on MDCK cells and their alpha-1B adrenoceptors contribute to the lack of inhibition of phenylephrine-induced down-regulation, we do not consider this likely because PMA-induced down-regulation was markedly inhibited in parallel experiments. Thus, our results strongly suggest that PKC does not play a major role in phenylephrine-promoted down-regulation of MDCK cell alpha-1B adrenoceptors. This is consistent with evidence in other cell systems that PKC activation can mimic the rapid agonist-induced alpha-1B adrenoceptor sequestration but that PKC is not involved in agonist-induced alpha-1 adrenoceptor sequestration (LATTION et al., 1994). Similarly, PKC activation can cause down-regulation of delta-opioid receptors expressed in HEK 293 cells or of angiotensin AT$_1$ receptors found in rat aortic smooth muscle cells, but PKC does not mediate agonist-induced down-regulation in these receptor systems (Lassegue et al., 1995; PEI et al., 1995).

KAGAYA et al. (1993) demonstrated that the calmodulin antagonist, W-7, prevents the desensitization of 5-hydroxytryptamine$_2$ receptors in C6BU-1 cells produced by a 4-hr incubation with serotonin. They concluded that Ca$^{2+}$/calmodulin protein kinase may participate in the homologous desensitization of phospholipase C-coupled receptors, such as the serotonin 5-hydroxytryptamine$_2$ receptors. Therefore, we tested whether Ca$^{2+}$/calmodulin protein kinase might mediate agonist-induced alpha-1B adrenoceptor down-regulation. However, our data with two distinct inhibitors of this pathway, W-7 and KT5926, demonstrate that this second messenger kinase is not involved in agonist-induced down-regulation of MDCK cell alpha-1B adrenoceptors.

In conclusion, we demonstrated that MDCK cell alpha-1B adrenoceptors can be down-regulated by the receptor agonist phenylephrine in a concentration- and time-dependent manner. Receptor down-regulation is accompanied by functional desensitization but does not involve altered expression of G$_{q,11}$, alpha subunits and is independent of formation of cyclooxygenase products. The alpha-1B adrenoceptor down-regulation also occurs in response to stimulation of purinergic receptors and by direct PKC activation. The latter treatment is more effective than phenylephrine in causing down-regulation. Nevertheless, PKC activation does not appear to mediate agonist-induced down-regulation, nor does the Ca$^{2+}$/calmodulin protein kinase mediate such down-regulation. Whether other kinases are involved in agonist-induced down-regulation of alpha-1B adrenoceptors and what their identity may be are not clear from the present study. A member of the family of GRK should be considered because alpha-1B adrenoceptors are substrates for several members of this group of kinases, including GRK2, GRK3 and GRK6 (PREMONET et al., 1995). Moreover, a role for GRK has been demonstrated in the rapid uncoupling and sequestration of alpha-1B adrenoceptors (DIVIANI et al., 1996). The GRK are also involved in agonist-induced down-regulation of delta-opioid receptors (PEI et al., 1995) and of angiotensin II receptors (OPPERMANN et al., 1996). Whether GRK indeed are involved in agonist-induced down-regulation of alpha-1B adrenoceptors remains to be studied.

**Acknowledgments**

The skillful technical assistance of Martina Michel-Reher is greatly appreciated.

**References**


Blue DR, Craig DA, Ransom JT, Camacho JA, Insel PA and Clarke DE (1994) Characterization of the alpha_{1}-adrenoceptor subtype mediating (H^{+})-arachidonic acid release and calcium mobilization in Madin-Darby canine kidney cells. J Pharmacol Exp Ther 268:1588–1596.


Daul AE, Wang XL, Michel MC and Brodde OE (1987) Arterial hypotension in chronic hemodialyzed patients. Br J Pharmacol 93:1, Klinikum, 45122 Essen, Germany. E-mail: martin.michel@uni-essen.de


