

Mechanisms of desensitization of the adrenocorticotropin response to arginine vasopressin in ovine anterior pituitary cells

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Abstract

Arginine vasopressin (AVP) stimulates adrenocorticotropin (ACTH) secretion from corticotroph cells of the anterior pituitary via activation of the V1b vasopressin receptor, a member of the G protein-coupled receptor (GPCR) family. Recently, we have shown that treatment of ovine anterior pituitary cells with AVP for short periods results in reduced responsiveness to subsequent stimulation with AVP. The aim of this study was to investigate mechanisms involved in this desensitization process. Among the GPCR family, rapid desensitization is commonly mediated by receptor phosphorylation, with resensitization being mediated by internalization and subsequent dephosphorylation of the receptors by protein phosphatases. Since desensitization of V1a vasopressin receptors is mediated by protein kinase C-mediated receptor phosphorylation, we investigated the involvement of this enzyme in desensitization of the ACTH response to AVP. Treatment of perfused ovine anterior pituitary cells with the specific protein kinase C (PKC) activator 1,2-dioctanoyl-*sn*-glycerol (300 μ M) did not induce any reduction in response to a subsequent 5-min stimulation with 100 nM AVP, despite potently stimulating ACTH secretion. Likewise, the results obtained using the PKC inhibitor Ro 31-8220 were not consistent

with involvement of PKC in AVP desensitization: 2 μ M Ro 31-8220 did not reduce the ability of a 10 nM AVP pretreatment to induce desensitization to a subsequent stimulation with 100 nM AVP. Pharmacologic blockade of receptor internalization by treatment with 0.25 mg/ml concanavalin A significantly impaired the ability of a 15-min pretreatment with 10 nM AVP to induce desensitization, rather than affecting resensitization. Treatment with 10 nM okadaic acid, an inhibitor of protein phosphatase 1 and 2A, had no effect on either resensitization or desensitization. In contrast, inhibition of protein phosphatase 2B (PP2B) with 1 μ M FK506 decreased the rate of resensitization: complete recovery from desensitization took 40 min, whereas in controls recovery was complete 20 min after termination of the pretreatment. These results indicate that desensitization of the ACTH response to AVP is not mediated by PKC-catalyzed phosphorylation, suggesting subtype-specific differences in the regulation of V1a and V1b vasopressin receptors. The data demonstrate that desensitization was dependent, at least in part, upon receptor internalization and that resensitization was dependent upon PP2B-mediated receptor dephosphorylation.

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Introduction

Arginine vasopressin (AVP) is an important physiologic regulator of adrenocorticotropin (ACTH) secretion from the anterior pituitary (Aguilera 1994). The effects of AVP on corticotroph cells of the anterior pituitary are mediated by activation of the V1b (also known as V3) vasopressin receptor, a phospholipase C (PLC)-coupled member of the G protein-coupled receptor (GPCR) superfamily (De Keyser *et al.* 1994). Recently, we have shown that treatment of perfused ovine anterior pituitary cells with AVP can cause desensitization of the ACTH response to a subsequent stimulation with AVP (Hassan *et al.* 2003). This desensitization occurred at concentrations and

durations of AVP treatment that were within the ranges of AVP pulses measured in the sheep portal circulation, suggesting that the desensitization process might play an important physiologic role in the regulation of ACTH secretion. Desensitization was found to be rapid (complete within 10 min of the onset of AVP treatment) and was readily reversible (resensitization was complete 40 min after the end of AVP treatment). The aim of the study reported here was to investigate molecular mechanisms involved in both desensitization and resensitization of the ACTH response to AVP.

The characteristics of the desensitization and resensitization processes give some clues as to mechanisms that might underlie them. Among the GPCR superfamily,

rapid and readily reversible desensitization is commonly a result of uncoupling of the receptor from its signaling pathway (Lohse 1993). This uncoupling is typically mediated by phosphorylation of the receptor by one or more protein kinases that, directly or indirectly, prevents the receptor from activating its signaling pathway. The effector kinases protein kinase A (PKA) and protein kinase C (PKC), the G protein-coupled receptor kinase (GRK) family and casein kinase 1 α (CK1 α) are all capable of phosphorylating GPCRs (Lohse 1993, Tobin *et al.* 1997).

A model has been proposed in which resensitization of desensitized, phosphorylated receptors is mediated by their internalization to an intracellular compartment (most likely the early endosome). The receptors are subsequently recycled back to the plasma membrane in a fully functional state. In this model, receptor phosphorylation is sufficient for desensitization, and internalization of the receptor is not required for reduced responsiveness (Ferguson *et al.* 1996). Experimental results consistent with this model have been reported for a variety of receptors, including the β_2 adrenergic receptor (Pippig *et al.* 1995) and the NK₁ neurokinin receptor (Garland *et al.* 1996). Two different types of protein phosphatase, PP2A and PP2B, have been implicated in the dephosphorylation of GPCRs (Pippig *et al.* 1995, Shih *et al.* 1999). Recent evidence suggests an alternative role for internalization for some members of the GPCR family, including the sst_{2B} somatostatin receptor (Beaumont *et al.* 1998) and the secretin receptor (Mundell & Kelly 1998). For these receptors, pharmacologic blockade of internalization prevents desensitization from occurring, suggesting that they must be sequestered from the cell membrane in order to be successfully uncoupled from their signaling pathways.

Vasopressin receptors found in the liver, brain and vasculature are of the V1a subtype (Birnbaumer 2000). Although these receptors are structurally distinct from V1b receptors (~45.5% amino-acid sequence homology in the human (De Keyser *et al.* 1994)), they share similar signaling properties: they have almost identical affinity for AVP (Jard *et al.* 1986), both are coupled to PLC (Birnbaumer 2000), and both undergo desensitization after exposure to AVP. In the case of the V1a receptor, desensitization appears to be mediated, at least in part, by PKC-mediated phosphorylation of the receptor (Caramelo *et al.* 1991, Gallo-Payet *et al.* 1991, Ancellin *et al.* 1997, Ancellin & Morel 1998). Given the pharmacologic similarities between the two receptor subtypes, the molecular mechanisms involved in their desensitization might be expected to be similar. Alternatively, in view of their distinct tissue distributions and the different concentrations and patterns of AVP they are exposed to, V1a and V1b receptors might be predicted to be regulated by different mechanisms of desensitization.

A second group of kinases that might be involved in desensitization of V1b receptors are the GRKs. However, while GRKs have been found to be involved in the

regulation of some PLC-coupled GPCRs, changes in the intracellular environment following activation of PLC appear somewhat unfavourable to GRK activity (Tobin *et al.* 1997). Thus, it has been suggested that in some cases alternative mechanisms might be involved in the regulation of PLC-coupled GPCRs. Recently, CK1 α was shown to phosphorylate the PLC-coupled m1 and m3 muscarinic receptors (Tobin *et al.* 1997, Waugh *et al.* 1999). This phosphorylation was agonist-dependent and was associated with a rapid desensitization of the cellular responses to stimulation.

Little information exists on the mechanisms involved in resensitization of the pituitary V1b receptor. While evidence indicates that the pituitary AVP receptor is internalized after AVP stimulation (Mogensen *et al.* 1988, Berrada *et al.* 2000), it is unclear what role this process might play in regulating the ACTH response to AVP. The role that protein phosphatases might play in resensitization is also unknown.

In this study we focused on investigating the mechanisms of desensitization and resensitization of the anterior pituitary ACTH response to AVP. Specifically, we used pharmacologic agents to investigate the roles of PKC, CK1 α , PP2A, PP2B and receptor internalization in these two processes. The results obtained were compared with those reported for desensitization of the V1a receptor.

Materials and Methods

Cell preparation

Pituitary glands were collected from a local abattoir in accordance with animal ethics laws of New Zealand and the guidelines of the University of Canterbury. Dispersed ovine anterior pituitary cells were prepared as previously described (Evans *et al.* 1985, Le Beau & Mason 1994). Briefly, whole pituitary glands were removed from sheep shortly after slaughter and placed into cold, sterile HEPES buffer (25 mM HEPES, 137 mM NaCl, 5 mM KCl, 10 mM glucose and 0.002% phenol red, pH 7.3) containing antibiotic and antimycotic agents (100 U penicillin/ml, 100 mg streptomycin/ml and 0.25 mg amphotericin B/ml). A suspension of dispersed cells from the combined anterior lobes of seven pituitaries was obtained following a combination of enzymatic (collagenase type II, 480 U/ml HEPES buffer, Sigma) and mechanical disruption. The dispersed cells were washed by centrifugation (300 g for 5 min at 4°C) and resuspension in HEPES buffer; after the final wash, they were resuspended in culture medium (Dulbecco's modified Eagle's medium (DMEM); Sigma) containing 3.7 mg NaHCO₃/ml, 584 mg L-glutamine/ml, nonessential amino acids, antibiotic/antimycotic agents (as above) and 25 mM HEPES at pH 7.3, and supplemented with 10% newborn calf serum (NCS) (Gibco Laboratories, Life Technologies, Grand Island, NY, USA). For perfusion experiments, 4.5 × 10⁶ cells were plated

into nonadherent plastic Petrie dishes (Labserv, Auckland, New Zealand) in 20 ml DMEM plus NCS.

Multicolumn perfusion experiments

The multicolumn perfusion system, as described previously and with modifications (Evans *et al.* 1985, 1996), allows for the simultaneous perfusion of up to 15 cell chambers or columns. Through the use of a solenoid switching system, various trains of peptide hormones and pharmacologic agents can be applied to the cells.

After overnight incubation, the cells from each culture plate were recovered by centrifugation, and the supernatant was replaced with 170 μ l slurry of preswollen Sephadex G-25 fine (Sigma) suspended at a ratio of 1:1.5 v/v in Krebs-Ringer (KR) solution (125 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 , 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 3.6 mM NaHCO_3 , 25 mM HEPES, 10 mM glucose and antibiotic/antimycotic agents as above). Slurries of Bio-Gel P2 (BioRad Laboratories, Hercules, CA, USA) (80 μ l suspended 1:1.5 v:v in KR solution) and Sephadex G-25 fine (80 μ l suspended 1:1.5 v:v in KR solution) were added sequentially to the columns to form a bed on which the mixture of cells and Sephadex was placed. Perfusion was commenced at a flow rate of 0.16 ml/min, with the perfusion medium (KR solution containing 0.05% alkali-treated casein and 0.005% ascorbate (Sigma); KR/ATC) and the cells being maintained at 37 °C by a water jacket surrounding the columns and the media reservoirs. Effluent from the columns was collected in 5- and 10-min fractions and frozen for subsequent measurement of ACTH concentration by radioimmunoassay.

During an experiment, cells were perfused with a 'basal' solution (that is, KR/ATC) and a 'test' solution containing AVP. The tubing carrying these solutions was connected to the pump tubing by a Y-junction. At any given time, one of these lines was clamped closed. Through the use of a solenoid-actuated switching system, the perfusion solution could be rapidly and precisely changed between the 'test' and 'basal' solutions. When the cells were perfused with 'basal' KR/ATC plus two separate 'test' solutions, the basal line was manually transferred between KR/ATC and the second 'test' solution.

A variety of different experimental protocols were used in perfusion experiments. While the protocol used depended on the aim of the particular experiment, the design of all experiments shared some common features. These are described below while the details of the specific treatment regimes used in individual experiments can be found in the Results section. Each of the 15 columns in the perfusion system was randomly assigned an experimental treatment. Typically, each of the different treatments was repeated in at least two columns. In all experiments, cells were perfused with 'basal' KR/ATC for at least 90 min at the beginning of the experiment, allowing them to

recover from transfer into the columns. During this period, ACTH secretion dropped to a consistently low level. Experimental treatments began at the end of this run-in period.

Radioimmunoassay

ACTH radioimmunoassay was performed as previously described (Evans *et al.* 1985). The antiserum used (rabbit antiporcine ACTH) was a gift from Dr Richard Donald, Christchurch Hospital (Christchurch, New Zealand), and the highly purified ovine ACTH, used for ^{125}I -radioiodination and assay standards, was a gift from Dr C H Li, Hormone Research Laboratory, University of California (San Francisco, CA, USA). Intra- and interassay coefficients of variation were 6.77% and 8.25%.

Data and statistical analysis

In perfusion experiments, the ability of a particular treatment to induce desensitization was assessed by measuring its effect on the response to a subsequent 5-min stimulation with 100 nM AVP. The magnitude of the response to each of these 'test' pulses was calculated as the total amount of ACTH released in the 20 min following commencement of the pulse, minus basal secretion (calculated using the mean value of the hormone concentration in the two or three fractions immediately preceding either the pulse or the pretreatment). Therefore, the term 'response' refers to stimulated ACTH secretion. For the purposes of analysis, the results from each perfusion column were treated as $n=1$. In general, for each treatment, there were two to three replicates (columns) per experiment (one-cell preparation), and there were two to three experiments. Data are reported as mean \pm S.E.M. Statistical significance was assessed by either Student's *t*-test or ANOVA, as indicated in the text. $P<0.05$ was considered significant.

Results

Effect of pretreatment with 1,2-dioctanoyl-sn-glycerol (DiC₈) on the ACTH response to AVP

To investigate whether PKC is involved in desensitization of the ACTH response to AVP, perfused cells were pretreated with the specific PKC activator DiC₈ to determine whether it induced desensitization to a subsequent stimulation with AVP. If PKC was involved in the desensitization process, pretreatment with DiC₈ would be expected to reduce the response to a subsequent stimulation with AVP. The experimental design was similar to that which we have previously used to investigate desensitization of the ACTH response to AVP (Hassan *et al.* 2003): perfused cells were stimulated with 5-min pulses of

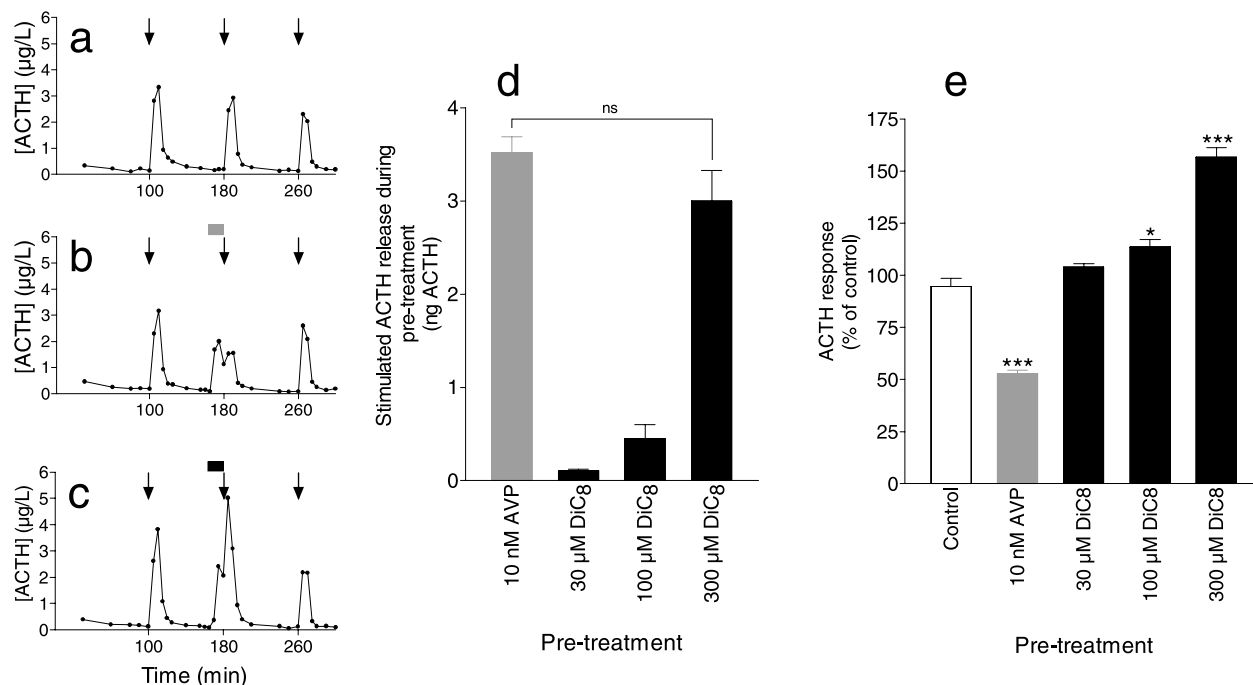


Figure 1 Treatment with the PKC activator DiC₈ stimulated ACTH secretion but did not induce desensitization. Panels a–c show representative data from perfusion columns in which cells were stimulated with 5-min pulses of 100 nM AVP after 100, 180 and 260 min of perfusion (100 nM AVP pulses are indicated by arrows). Results from a control column (that is, no pretreatment) are shown in panel a. Results from columns in which the second 100 nM AVP pulse was immediately preceded by a 15-min pretreatment with 10 nM AVP (indicated by gray bar) or 300 μM DiC₈ (indicated by black bar) are shown in panels b and c respectively. DiC₈-stimulated ACTH secretion during the pretreatment period was concentration dependent, with 300 μM DiC₈ stimulating ACTH secretion at a level equivalent to that stimulated by 10 nM AVP (d; data are mean ± S.E.M., n=6 for each treatment). Despite potentially stimulating ACTH secretion, pretreatment with DiC₈ did not induce desensitization to a subsequent AVP stimulation (e). Data shown are the responses, expressed as a percentage of control, to a 5-min, 100 nM AVP pulse after pretreatment for 15 min with the indicated solutions. Data are mean ± S.E.M. (n=5–6 for each treatment). Asterisks indicate a statistically significant difference between the results observed and 100% (t-test; *P<0.05; ***P<0.001).

AVP after 100, 180 and 260 min of perfusion, resulting in three peaks of ACTH secretion (Fig. 1a). Since the mean of the responses to the first and third pulses is no different to the response to the second pulse (n=9; NS, Student's *t*-test), it is assigned as control (100%). To induce desensitization, the second pulse was immediately preceded by a 15-min pretreatment with either 10 nM AVP (Fig. 1b) or DiC₈ at concentrations of either 30, 100 or 300 μM (Fig. 1c). The effect of pretreatment on the ACTH response to AVP was assessed quantitatively for each perfusion column by expressing the response to the pretreated (second) pulse as a percentage of the mean of the responses to the first and third pulses (that is, control). Secretion of ACTH during pretreatment with DiC₈ was concentration dependent (Fig. 1d). Treatment with 30 μM DiC₈ caused a small but significant increase in ACTH secretion above basal (n=6, P<0.01, *t*-test). When the concentration was increased to 300 μM, stimulated ACTH secretion was not significantly different from that observed during pretreatment with 10 nM AVP (one-way ANOVA with Dunnett's test). Despite stimulating

ACTH secretion at a level equivalent to AVP, pretreatment with DiC₈ did not induce desensitization. In contrast to pretreatment with 10 nM AVP (which caused a reduction in response of 48.6 ± 1.6% (n=6, P<0.0001, *t*-test)), the ACTH response to AVP was increased in a concentration-dependent manner after pretreatment with DiC₈ (Fig. 1e). Pretreatment with 100 μM and 300 μM DiC₈ caused 9.9 ± 3.7% (n=6, P<0.05, *t*-test) and 51.3 ± 4.4% (n=6, P<0.0001, *t*-test) increases in response respectively.

Effect of pretreatment with the specific PKC inhibitor Ro 31-8220 on desensitization of the ACTH response to AVP

The involvement of PKC in desensitization of the ACTH response to AVP was investigated further with the potent and selective PKC inhibitor Ro 31-8220 (or, more fully, 2-{1-[3-(amidino-thio)propyl]-1H-indol-3-yl}-3-(1-methylindol-3-yl)-maleimide methanesulfonate) (Davis *et al.* 1992). If PKC were involved in the desensitization

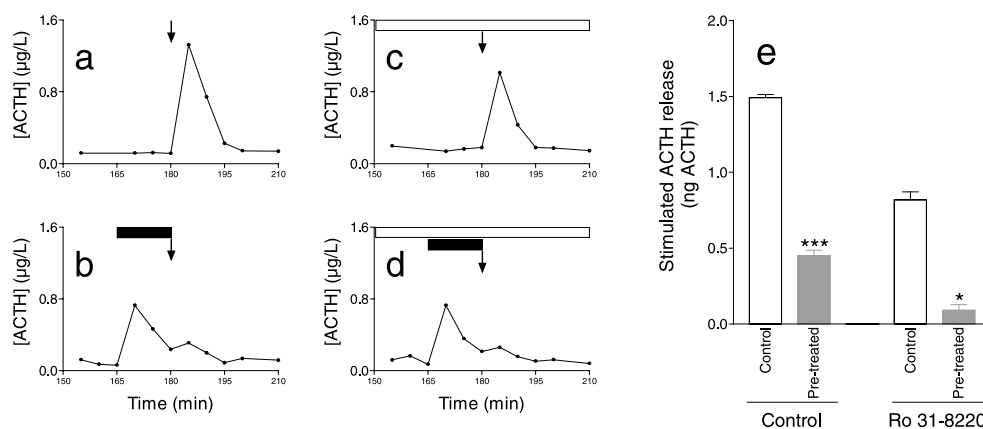


Figure 2 Treatment with the PKC inhibitor Ro 31-8220 does not prevent AVP-induced desensitization. Treatment with a 100 nM AVP pulse (indicated by arrows) resulted in a peak of ACTH secretion both in the absence (a) and presence (c) of 2 µM Ro 31-8220. Pretreatment with 10 nM AVP for 15 min (indicated by black bars) resulted in a reduction in response to a subsequent stimulation with 100 nM AVP, both in the absence (b) and presence (d) of Ro 31-8220. Representative data from four perfusion columns are shown. Data reflect the ACTH secreted in response to a 100 nM AVP pulse both with and without pretreatment with 10 nM AVP for 15 min, and in the presence (gray bars) and absence (open bars) of Ro 31-8220. Data are mean \pm S.E.M. ($n=3-4$ for each treatment). Asterisks indicate statistically significant difference between the pretreated and control value for each pair of responses (t -test; * $P<0.05$; *** $P<0.001$).

process, treatment with Ro 31-8220 would be expected to impair the ability of AVP pretreatment to induce desensitization. In preliminary studies, we found that the effects of Ro 31-8220 were not readily reversible. Thus, it was not possible to analyze desensitization as described above, using the first and third pulses as controls in a three-pulse protocol. To induce desensitization, a 5-min 100 nM AVP pulse at 180 min was preceded by a 15-min pretreatment with 10 nM AVP. This AVP pretreatment caused a significant reduction ($69.8 \pm 2.4\%$; $n=3$, $P<0.05$, t -test) in response compared with the response to a similar 100 nM AVP pulse in control columns (that is, not pretreated) (Fig. 2a and b). To assess the effect of PKC inhibition on desensitization of the ACTH response to AVP, cells were treated with 2 µM Ro 31-8220 from 90 min of perfusion until the completion of the experiment. During treatment with Ro 31-8220, the ACTH response to the 100 nM AVP pulse was reduced by $45.1 \pm 3.5\%$ ($n=7$, $P<0.0001$, t -test) compared with controls that were not treated with Ro 31-8220 (Fig. 2; compare panels c and a). However, despite being able to inhibit ACTH secretion, treatment with Ro 31-8220 did not reduce the extent of desensitization observed. After pretreatment with 10 nM AVP in combination with Ro 31-8220, the response to a subsequent stimulation with 100 nM AVP was reduced by $88.8 \pm 4.5\%$ ($n=4$, $P<0.01$, t -test) compared with the corresponding control (that is, Ro 31-8220-treated but not 10 nM AVP-pretreated) (Fig. 2; compare panels d and c). Thus, rather than reducing the extent of AVP-induced desensitization,

Ro 31-8220 significantly increased the desensitization caused by AVP pretreatment ($n=7$, $P<0.05$, t -test). Results are summarized in Fig. 2e.

Effect of pretreatment with CK1-7 on desensitization of the ACTH response to AVP

The role of CK1 α in the regulation of AVP-stimulated ACTH secretion was investigated with the potent and selective casein kinase 1 inhibitor CK1-7 (*N*-[2-aminoethyl]-5-chloroisoquinoline-8-sulfonamide) (Chijiwa *et al.* 1989). The involvement of CK1 α in desensitization of the ACTH response to AVP was investigated by testing the effect of treatment with CK1-7 on the ability of AVP pretreatment to induce desensitization. If CK1 α were involved in desensitization, the presence of CK1-7 would be expected to reduce the extent of desensitization induced by AVP pretreatment. Pretreatment with 10 nM AVP for 25 min before the second 100 nM AVP pulse in a three-pulse protocol caused a reduction in response of $49.0 \pm 3.1\%$ ($n=9$, $P<0.001$, t -test) (Fig. 3a) compared with controls. To investigate the involvement of CK1 α in desensitization, the second 100 nM AVP pulse was immediately preceded by a 30-min treatment with CK1-7 at concentrations of 10, 30 or 100 µM (that is, for 5 min before the pretreatment and then concurrently with it for 25 min) (Fig. 3b-d). Treatment with CK1-7 at all three concentrations had no effect on AVP-induced desensitization, with the reduction in response induced by AVP pretreatment not being affected

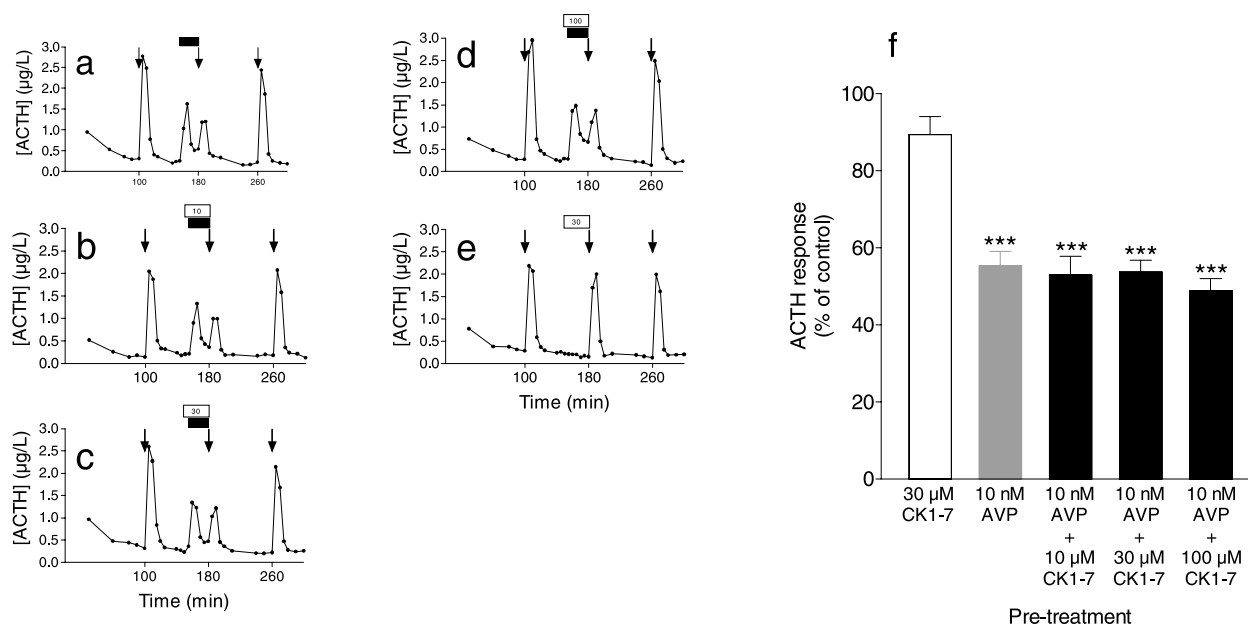


Figure 3 Treatment with the CK1 α inhibitor CK1-7 did not prevent AVP-induced desensitization of the ACTH response to a subsequent stimulation with 100 nM AVP. Data in panels a–e are from single representative perfusion columns in which the second 100 nM AVP pulse has been preceded by treatment with 10 nM AVP alone (a) and 10 nM AVP in combination with either 10 μ M CK1-7 (b), 30 μ M CK1-7 (c) or 100 μ M CK1-7 (d), and with 30 μ M CK1-7 alone (e). Black bars indicate the 10 nM AVP pretreatment, and open bars indicate the CK1-7 pretreatment, while the 100 nM AVP pulses are indicated by arrows. Summarized results are shown in panel f. Data shown are the responses, expressed as a percentage of control, to a 5-min, 100 nM AVP pulse after pretreatment with the indicated solutions. Data are mean \pm S.E.M. ($n=6-9$ for each treatment). Asterisks indicate a statistically significant difference between the results observed and 100% (t -test; $***P<0.001$).

by any of these concentrations of CK1-7 (Dunnett's test). After 30-min pretreatment with CK1-7 (30 μ M) alone, the response to the second AVP pulse was not significantly altered compared with the controls ($n=6$, NS, t -test) (Fig. 3e). Results are summarized in Fig. 3f.

Treatment with CK1-7 both alone and in combination with AVP had no effect on ACTH secretion. During pretreatment with 30 μ M CK1-7 alone, ACTH secretion was not significantly increased (0.22 ± 0.17 ng ACTH above basal during the entire 30-min CK1-7 treatment; $n=3$, NS, t -test). Similarly, treatment with 10 nM AVP in combination with CK1-7 had no effect on stimulated ACTH secretion during the 25-min 10 nM AVP pretreatment (10 nM AVP alone: 2.46 ± 0.07 ng ACTH; 10 nM AVP + 100 μ M CK1-7: 2.45 ± 0.09 ng ACTH; $n=6$, NS, t -test).

Effect of blockade of receptor internalization on the regulation of the ACTH response to AVP

The involvement of receptor internalization in the regulation of the ACTH response to AVP was investigated with the lectin concanavalin A (ConA) to block receptor internalization (Pippig *et al.* 1995, Trincavelli *et al.* 2000). The experimental protocol employed was based on

that which we have previously used to investigate resensitization of the ACTH response to AVP (Hassan *et al.* 2003). Perifused cells were stimulated with a single, 5-min pulse of 100 nM AVP after 160 min of perfusion, resulting in a characteristic peak of ACTH secretion. To induce desensitization, this pulse was immediately preceded by 15-min pretreatment with 10 nM AVP. To observe resensitization, a recovery period of either 20 or 40 min was allowed between the end of the pretreatment and the start of the pulse. The extent of desensitization was assessed quantitatively by expressing the response to the 100 nM AVP pulse after pretreatment as a percentage of the response of control cells (which were not pretreated) to 100 nM AVP pulses.

When cells were not treated with ConA, pretreatment with 10 nM AVP for 15 min immediately prior to the 100 nM AVP pulse caused a reduction in response of $58.4 \pm 4.4\%$ ($P<0.001$, one-way ANOVA with Bonferroni's test) compared with control (Fig. 4a and b). When a recovery period was allowed between the pulse and the pretreatment, resensitization occurred (Fig. 4c and d). After a 20-min recovery period, no significant desensitization remained (NS, Bonferroni's test); that is, resensitization was complete. When cells were treated with ConA (0.25 mg/ml) for 70 min prior to the 100 nM

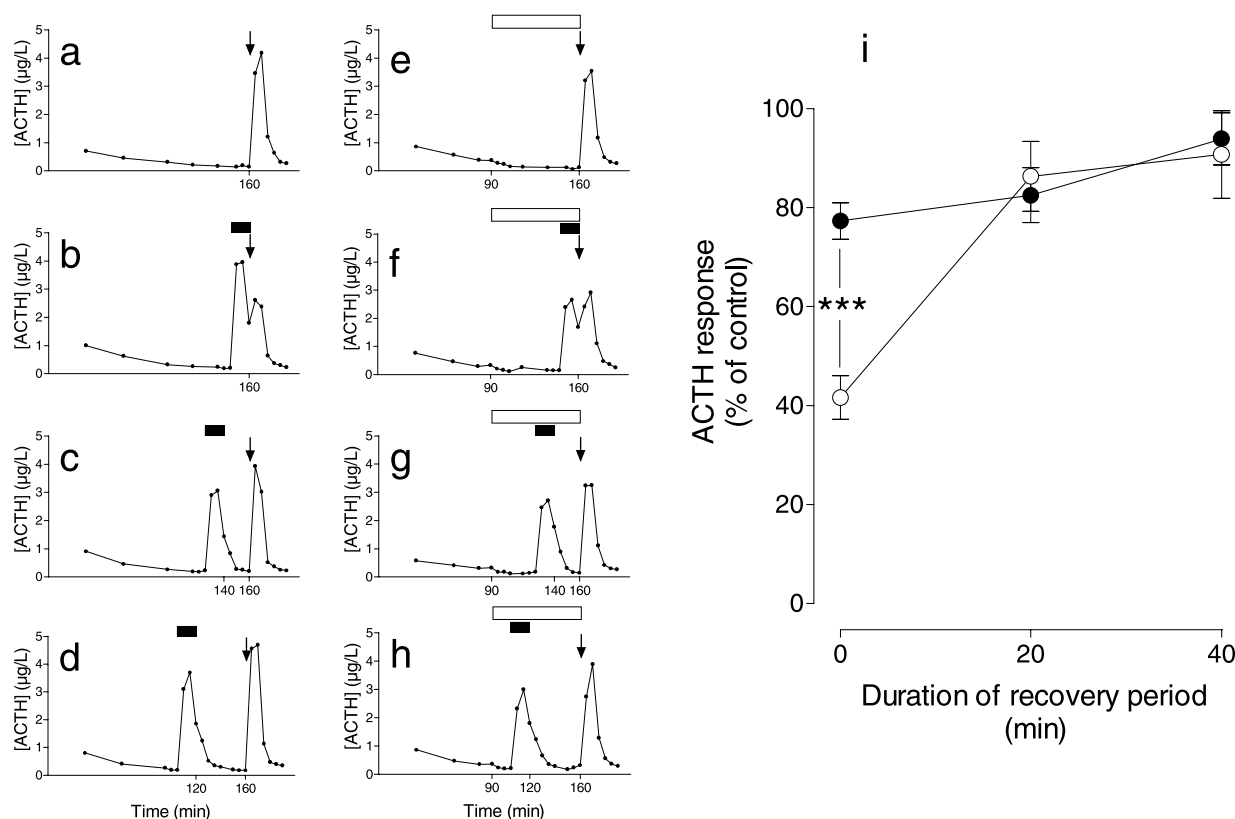


Figure 4 Effect of treatment with 0.25 mg/ml ConA on the ACTH response to AVP. Representative data from eight perfusion columns are shown in panels a–h. Cells were treated with a 5-min pulse of 100 nM AVP after 160 min of perfusion (indicated by arrows). To induce desensitization, this AVP pulse was immediately preceded by 15-min pretreatment with 10 nM AVP (indicated by black bars; b and f). When recovery periods of 20 or 40 min were allowed between the pretreatment and the pulse, resensitization occurred (c, d, g and h). The effect of pharmacologic blockade of receptor internalization on regulation of the ACTH response to AVP was investigated by treating cells with 0.25 mg/ml ConA for 70 min prior to the 100 nM AVP pulse (e–h; ConA treatment indicated by open bars). Combined results from columns in which ConA was present (closed symbols) and absent (that is, controls; open symbols) are shown in panel i. The ACTH response after pretreatment, as a percentage of control, is plotted against the duration of the recovery period. Data are mean \pm S.E.M. ($n=5-8$ for each treatment). Asterisks indicate a statistically significant difference in the extent of desensitization observed between the control and ConA-treated cells (Bonferroni's test; $***P<0.001$).

AVP pulse (that is, from 90 to 160 min), AVP pretreatment was still able to induce desensitization, but it was significantly reduced compared with the desensitization observed in the absence of the lectin. When the AVP pretreatment immediately preceded the 100 nM AVP pulse, the response was reduced by $22.7 \pm 3.7\%$ ($P<0.05$, Bonferroni's test) compared with the response of cells that were not pretreated with AVP, but were treated with ConA (Fig. 4e and f). This effect of ConA on the ability of AVP pretreatment to induce desensitization was statistically significant ($P<0.001$, Bonferroni's test). After recovery periods of 20 and 40 min, there was no significant difference between the extent of desensitization observed in control and ConA-treated cells (Fig. 4g and h). Results are summarized in Fig. 4i. Treatment with ConA had no significant direct effect on either basal or AVP-stimulated ACTH secretion.

Effect of inhibition of protein phosphatases on resensitization of the ACTH response to AVP

The role of PP2A and PP2B in resensitization of the ACTH response to AVP was assessed by pharmacologic inhibition of these two enzymes. A design similar to that used to investigate the role of internalization was employed, except that the cells were treated with the appropriate phosphatase inhibitor for 70 min prior to the 100 nM AVP pulse rather than with ConA.

FK506 is a macrocyclic lactone derived from *Streptomyces* sp. that specifically inhibits PP2B (Schreiber 1991). Treatment with 1 μ M FK506 did not alter the magnitude of desensitization induced by pretreatment with 10 nM AVP for 15 min ($54.6 \pm 6.4\%$ in the presence of FK506 compared with $53.7 \pm 3.5\%$ in controls not treated with the inhibitor (NS, Bonferroni's test)). However, treatment

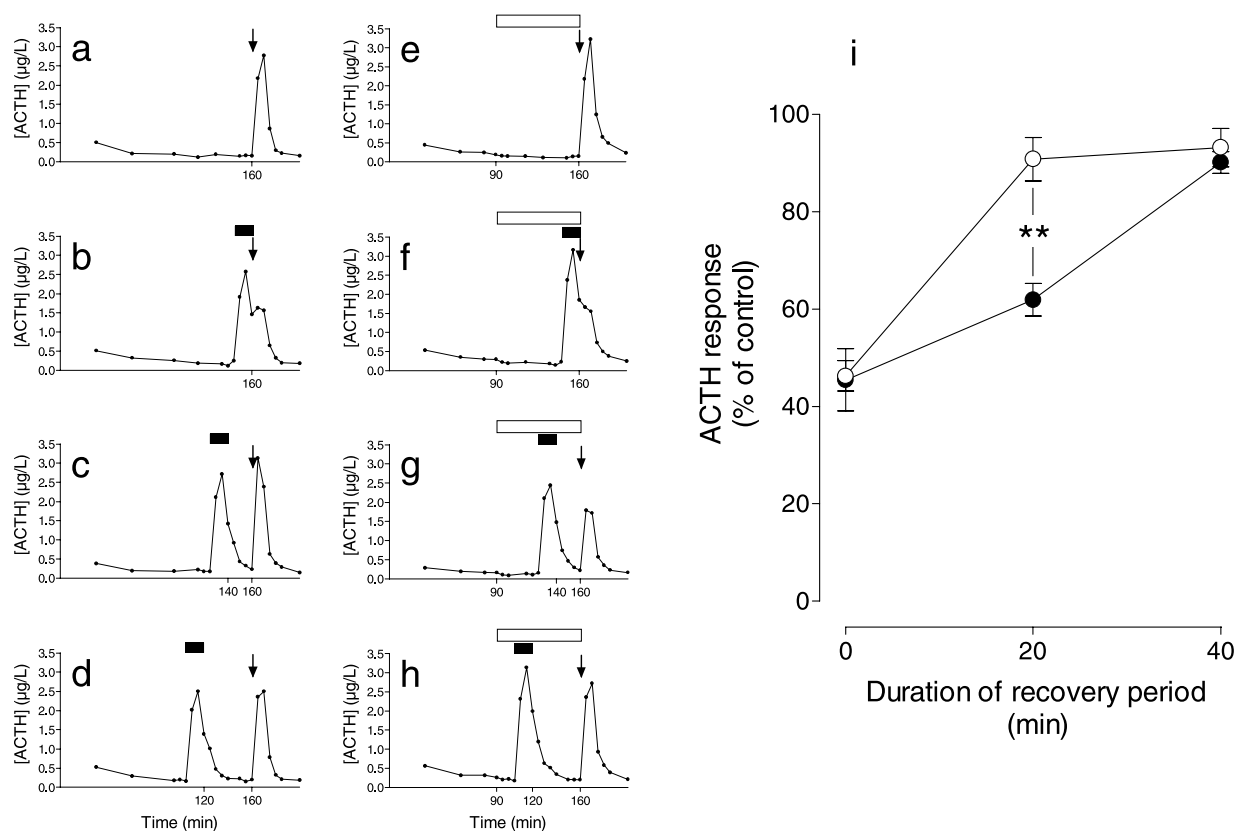


Figure 5 Treatment with 1 μM FK506 attenuates resensitization of the ACTH response to AVP. Representative data from eight perfusion columns are shown in panels a–h. The experimental protocol used was identical to that described in Fig. 3, except that, instead of ConA, cells were treated with 1 μM FK506 for 70 min immediately prior to the 100 nM AVP pulse (e–h). Combined results from columns in which FK506 was present (closed symbols) and absent (that is, controls; open symbols) are shown in panel i. The ACTH response following pretreatment, as a percentage of control, is plotted against the duration of the recovery period. Data are mean \pm S.E.M. ($n=4$ –10 for each treatment). Asterisks indicate a statistically significant difference between the extent of desensitization observed between the control and FK506-treated cells (Bonferroni's test; $**P<0.01$).

with FK506 decreased the rate of resensitization. In the absence of FK506, recovery from desensitization was complete after 20 min ($9.2 \pm 5.1\%$ less than control; NS, Bonferroni's test). In contrast, when cells were treated with 1 μM FK506, a significant desensitization still remained at 20 min ($38.1 \pm 3.3\%$ less than control; $P<0.001$, Bonferroni's test). Resensitization was not complete until 40 min. Representative data from individual columns in which cells were treated with 1 μM FK506 are shown in Fig. 5e–h, and summarized results are shown in Fig. 5i.

In addition to this effect on the rate of resensitization, treatment with FK506 also caused a small but reproducible increase in the ACTH response to AVP pretreatment. Treatment with FK506 at concentrations of either 50 nM or 1 μM increased the ACTH response to the 15-min 10 nM AVP pretreatment (Fig. 6). Treatment with FK506 had no significant effect on either the ACTH response to the 100 nM AVP pulse or basal ACTH secretion.

Okadaic acid (OA) potently inhibits the activity of both protein phosphatase 1 (PP1) and PP2A (Herzig & Neumann 2000). PP2A is completely inhibited by 1 nM OA whereas PP1 is unaffected by this concentration, its 50% inhibitory concentration (IC_{50}) being 10–15 nM (Cohen *et al.* 1989). PP2B is relatively unaffected by OA ($\text{IC}_{50}=3600$ nM) (Bialojan & Takai 1988). Treatment with 10 nM OA for 70 min prior to the 100 nM AVP pulse had no effect on the pattern of desensitization and resensitization observed after pretreatment with 10 nM AVP for 15 min. Results are summarized in Fig. 7. There was no evidence of an effect of treatment with 10 nM OA on either basal or AVP-stimulated ACTH secretion.

Discussion

This study has shown that the mechanisms underlying desensitization of the V1b vasopressin receptor differ from

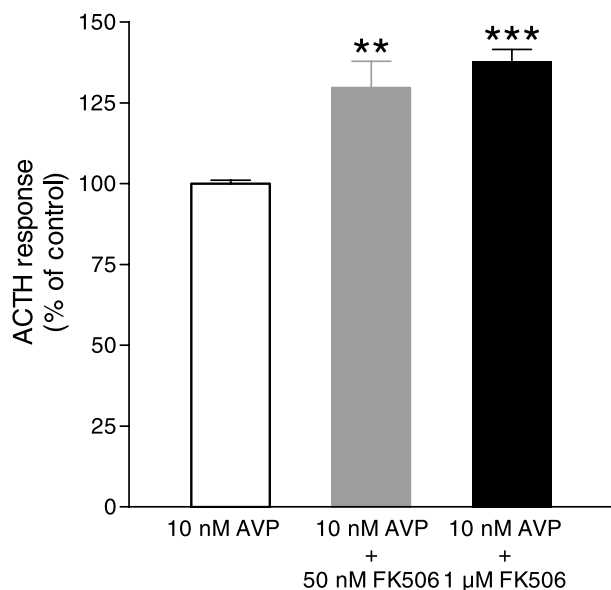


Figure 6 Effect of treatment with FK506 on the ACTH response to the 15-min pretreatment with 10 nM AVP. The ACTH response to the AVP pretreatment during treatment with 50 nM (gray bar) and 1 μM FK506 (black bar) is expressed as a percentage of the response to pretreatment with AVP alone in control columns (open bar). Data are from columns in which the 10 nM AVP pretreatment began 15 min after the commencement of the FK506 treatment (Fig. 4d and h). Asterisks indicate a statistically significant difference between the results observed with FK506 treatment and the control (Bonferroni's test; ** $P < 0.01$; *** $P < 0.001$). Data are mean \pm S.E.M. ($n = 2-4$ for each treatment).

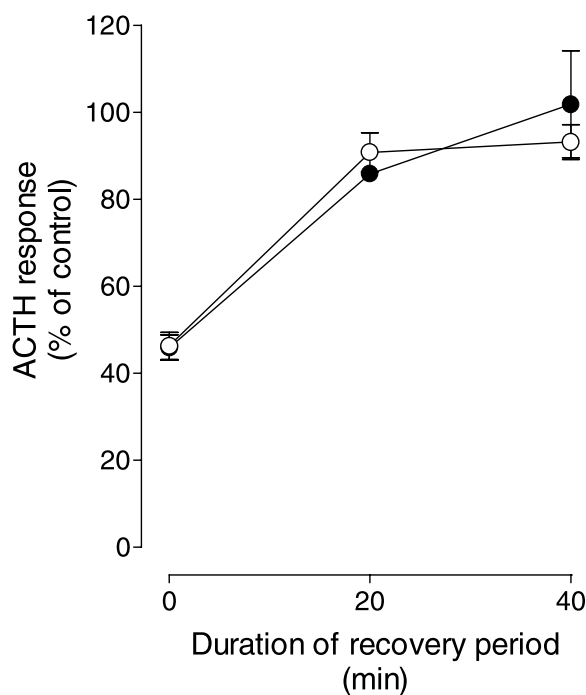


Figure 7 Effect of treatment with 10 nM okadaic acid (OA) on desensitization and resensitization of the ACTH response to AVP. The ACTH response after pretreatment with 10 nM AVP for 15 min is plotted against the duration of the recovery period. Combined results from columns in which OA was present (closed symbols) and absent (that is, controls; open symbols) are shown. Data are mean \pm S.E.M. ($n = 2-10$ for each treatment).

those of the V1a receptor. These differences are likely to be physiologically important in the regulation of responsiveness to AVP in different tissues.

No evidence was found for the involvement of either CK1 α or PKC in desensitization of the ACTH response to AVP. Despite being able potently to activate PKC, treatment with DiC $_8$ was not capable of inducing desensitization. In fact, it markedly increased the response to subsequent AVP stimulation. Likewise, the results obtained with the PKC inhibitor Ro 31-8220 were not consistent with involvement of PKC in AVP desensitization. Despite being able to reduce ACTH secretion significantly, treatment with Ro 31-8220 did not reduce the extent of desensitization induced by pretreatment with 10 nM AVP. Taken together, these results suggest that, while PKC plays an important role in the regulation of ACTH secretion, it is not involved in desensitization.

The lack of involvement of PKC in desensitization of the ACTH response to AVP suggests AVP receptor subtype-specific differences in mechanisms of desensitization of vasopressin receptors: the V1a vasopressin receptor, which is expressed in the liver, brain and vasculature (Birnbauer 2000), is desensitized via PKC-mediated receptor phosphorylation (Caramelo *et al.* 1991, Gallo-Payet *et al.* 1991, Ancellin *et al.* 1997, Ancellin &

Morel 1998). Differences in the mechanisms of desensitization might play an important role in the regulation of responsiveness to AVP in different tissues. The patterns and maximal concentrations of AVP secretion into the pituitary portal circulation are quite different from those in the peripheral circulation (Caraty *et al.* 1990). AVP is released into the portal blood in a highly pulsatile manner and reaches relatively high concentrations, that is, up to ~ 6 nM during insulin-induced hypoglycaemia in sheep (Caraty *et al.* 1990). In contrast, changes in AVP concentration in the peripheral circulation are slow and of low magnitude; for example, after hypertonic saline infusion into ewes, there is a steady increase in plasma AVP concentration from ~ 2 to ~ 7 pM over a period of 90 min (Keller-Wood 1994). In the β -adrenergic receptor system, GRK-mediated desensitization is very rapid and requires relatively high agonist concentrations, suggesting that this process might be particularly important in environments such as the synaptic cleft, where agonist concentrations increase rapidly to high concentrations (Roth *et al.* 1991). Correspondingly, the PKA-mediated desensitization of the β -adrenergic receptor is slower and occurs at lower agonist concentrations, suggesting that it is better suited to the regulation of responsiveness at nonsynaptic receptors

(Roth *et al.* 1991). Similarly, differential desensitization of the V1a and V1b receptors may allow for appropriate regulation of the responsiveness to AVP in different tissues where different concentrations and patterns of AVP exposure occur. Thus, differential desensitization of the V1a and V1b receptors provides a plausible explanation for the existence of two receptors with otherwise similar signaling properties.

The reduction in desensitization caused by inhibition of receptor endocytosis indicates that desensitization of the ACTH response to AVP is mediated, at least in part, by receptor internalization. It is of interest that, at the concentration used, ConA only partially inhibited the ability of AVP pretreatment to induce desensitization. This concentration (0.25 mg/ml) has been shown, in some instances, to inhibit completely internalization of a variety of GPCRs (Pippig *et al.* 1995), while in others it only partially inhibited internalization (Trincavelli *et al.* 2000). If receptor internalization was completely inhibited by ConA in the perfused anterior pituitary cell system used in these experiments, the remaining desensitization must have been the result of another desensitization process or processes. Alternatively, the ConA treatment used may have only partially inhibited receptor internalization and as a result only partially inhibited the development of desensitization.

While neither PKC nor CK1 α appears to play a role in desensitization to AVP, the effects of selective phosphatase inhibition on resensitization strongly suggest that protein phosphorylation and dephosphorylation are involved in desensitization and resensitization of the ACTH response to AVP. While no evidence could be found for the involvement of PP2A in resensitization, the attenuation in the rate of resensitization seen during FK506 treatment indicates a role for PP2B in recovery of the ACTH response to AVP. The role of PP2B in resensitization has been most extensively investigated for the β_2 -adrenergic receptor, where the enzyme appears to mediate resensitization by reversing GRK2-mediated phosphorylation of the receptor (Shih *et al.* 1999). It appears likely that PP2B plays a similar role in the regulation of the ACTH response to AVP, mediating the dephosphorylation of V1b receptors phosphorylated during desensitization. However, because a wide variety of membrane and cytosolic proteins are phosphorylated during AVP stimulation (Liu 1994, Liu *et al.* 1994), the possibility that dephosphorylation of proteins other than the AVP receptor is involved in resensitization cannot be excluded.

If desensitization is mediated by V1b receptor phosphorylation, the question remains as to which intracellular protein kinase is involved in this process. Perhaps the best remaining candidates are members of the GRK family. Neill *et al.* (1998) have shown that GRK2, GRK3, GRK6 and β -arrestins are expressed in rat anterior pituitary cells, demonstrating that, at least in this species, pituitary cells have the intracellular machinery necessary for regulation of

the V1b receptor by GRKs. Furthermore, recent evidence has shown that the V1b receptor can interact with GRKs (Berrada *et al.* 2000). The investigation of the involvement of GRKs in the desensitization of the ACTH response to AVP is somewhat difficult in primary cell cultures. At present, no highly specific inhibitors of GRK activity exist, and other, more specific techniques, such as expression of dominant negative mutant GRKs (Pitcher *et al.* 1998), or reducing GRK expression with antisense oligonucleotide sequences (Shih & Malbon 1994), are either extremely difficult or impossible to use in primary cell cultures. Although in this respect use of primary cultures is disadvantageous, it has one distinct advantage. Recent evidence has shown that the level of expression of GPCRs can affect their regulation (Spurney 1998) and that regulation of GPCRs can vary depending upon the nature of the cellular environment in which they are expressed (Ferguson 2001). In the anterior pituitary cell system used in this research, the expression of all components of the signaling pathway was under physiologic regulation. As such, the mechanisms which were found to be involved in desensitization of the ACTH response to AVP are very likely to be physiologically relevant and not artifacts of the system being investigated.

If the role of PP2B in resensitization of the ACTH response to AVP is to reverse phosphorylation of the V1b receptor (by an as yet undetermined protein kinase), the question of what role this phosphorylation might play in desensitization is raised. Receptor phosphorylation is usually, directly or indirectly, sufficient to induce desensitization; that is, internalization of the receptor is not required for its desensitization (Lohse *et al.* 1990). However, we have shown that desensitization of the ACTH response to AVP can, to a large extent, be inhibited by pharmacologic blockade of receptor internalization, indicating that receptor sequestration is important in this desensitization process. One possibility is that there are dual mechanisms of desensitization, with receptor phosphorylation mediating the proportion of desensitization that could not be inhibited by blockade of internalization with ConA. Another possible role for receptor phosphorylation in the regulation of the V1b receptor is as a signal that acts to promote receptor sequestration. These two hypotheses for a role of receptor phosphorylation in desensitization of the pituitary AVP receptor are not necessarily mutually exclusive. Receptor phosphorylation and subsequent binding of β -arrestins could impair, but not completely prevent, coupling of the V1b receptor to G $_{q/11}$, with subsequent phosphorylation-dependent internalization of the receptor resulting in full desensitization.

In summary, we have shown that desensitization of the ACTH response to AVP depends to a large extent, if not entirely, upon receptor internalization. As such, the pituitary V1b AVP receptor joins the small group of GPCRs for which receptor internalization is important in desensitization. Additionally, the activity of PP2B plays an

important role in resensitization, most likely by dephosphorylating pituitary AVP receptors that had been phosphorylated during the desensitization process. Desensitization was not the result of PKC-mediated phosphorylation, indicating that the mechanisms involved in desensitization of the pituitary V1b receptor are different from those involved in desensitization of the V1a receptor.

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