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Demonstration of processing and recycling of biologically active V_1 vasopressin receptors in vascular smooth muscle

([^{3}H]arginine vasopressin/processing/recycling/biological activity)

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ABSTRACT **The present study examines the binding and postbinding cellular processing and recycling of the V_1 arginine vasopressin (AVP) receptor in cultured vascular smooth muscle cells (VSMCs). The surface binding of AVP to VSMCs was temperature dependent and reached equilibrium within 60 min at 4°C. Displacement studies with unlabeled AVP or a specific V_1 AVP antagonist revealed a single class of V_1 receptors (B_{max}, 1.99 nmol per mg of protein; K_d, 2.15 nM). Incubation of VSMCs with unlabeled 10 nM AVP to promote receptor internalization resulted in a time- and temperature-dependent loss of AVP surface binding. At 37°C, maximum loss of binding sites (65%) occurred within 20 min. Recovery of AVP binding occurred rapidly ($t_{1/2}$, 15–20 min at room temperature) and was uninfluenced by inhibiting protein synthesis with cycloheximide. Pretreating VSMCs with chloroquine prevented AVP receptor recycling, indicating that the AVP–receptor complex requires endosomal processing. The biological competence of the recycled AVP receptor was shown by AVP-induced Ca^{2+} uptake. The results of these studies therefore indicate that, after surface binding, the AVP–receptor complex internalizes and dissociates in an endosomal compartment. It is demonstrated that in VSMCs biologically active V_1 AVP receptors recycle back to the cell surface, thus attenuating the loss of AVP surface binding sites.**

The vascular action of arginine vasopressin (AVP) is important for maintenance of systemic blood pressure in numerous pathophysiologic states (1). Although the mechanism whereby AVP induces the contraction of vascular smooth muscle cells (VSMCs) has been extensively investigated (2), there is little information available regarding the fate of AVP and its V_1 receptor after VSMC surface binding and internalization. In view of the importance of the vascular action of AVP, an understanding of AVP receptor processing and potential recycling by VSMCs has considerable physiological relevance as an important determinant of the rate of termination of ligand–receptor interaction and the resulting biological response.

Studies of a variety of different ligands have shown that the ligand–receptor complex may be processed by one of at least four different pathways. (*i*) The complex may be internalized by the cell with subsequent dissociation of the ligand from the receptor. The ligand is then targeted for lysosomal degradation and the free receptor recycles back to the cell surface (3). (*ii*) The ligand–receptor complex may be internalized but recycled to the cell surface intact and then externalized (4). (*iii*) The ligand–receptor complex may be internalized and then undergo lysosomal degradation, thus requiring new receptor synthesis (5). (*iv*) The complex may be transcytosed through the cell without lysosomal processing or recycling (6).

To date, the processing of the V_1 AVP–receptor complex in VSM has received little attention. A recent publication demonstrated that fluorescence-labeled AVP binds to A10 cells (a VSMC line) and then the AVP–receptor complex internalizes (7). The authors suggested, however, that the V_1 receptor does not recycle. However, the fluorescence-labeled ligand may have dissociated from the receptor.

In view of the importance of the vascular action of AVP and the lack of data concerning the processing and potential recycling of the AVP–receptor complex in vascular tissue, the present study was undertaken to determine the kinetics of AVP surface binding and the subsequent cellular processing of the ligand receptor complex in cultured rat VSMCs.

MATERIALS AND METHODS

VSMC Culture. Rat (Sprague–Dawley) aortic VSMCs were isolated and cultured as described (8). Cells were grown in minimal essential medium supplemented with 2 mM L-glutamine, 2 g of $NaHCO_3$ per liter, 60 mg of penicillin per liter, 135 mg of streptomycin per liter, and 10% fetal calf serum. Every 5–10 days, the cells were passaged. For all experiments, passage 1–6 cells were used.

[^{3}H]AVP Binding to VSMCs. Binding studies were performed as described in detail (8). VSMCs were washed with ice-cold binding buffer (119.2 mM NaCl/3.0 mM KCl/1.2 mM $MgSO_4$/1.0 mM $CaCl_2$/1.2 mM KH_2PO_4/10 mM glucose/10 mM Hepes/0.1% bovine serum albumin) and then incubated with 2 nM [^{3}H]AVP (specific activity, 67.7 μCi/mmol; 1 Ci = 37 GBq) with or without unlabeled AVP for 90 min at 4°C. Binding was terminated by aspirating the solution and the unbound radioligand was removed by washing with ice-cold binding buffer. The cells were then solubilized in 1 ml of 0.1% SDS and 0.1 M NaOH, and the radioactivity associated with cells was determined by scintillation counting (Packard Tri-Carb 460C). Specific binding of AVP was defined as total binding (2 nM [^{3}H]AVP) minus nonspecific binding (2 nM [^{3}H]AVP and 1 μM unlabeled AVP). Each binding assay was performed in triplicate. A 50-μl aliquot of the solubilized cells was assayed for protein content (9).

AVP Receptor Internalization Studies. *AVP receptor internalization.* VSMCs were incubated with 2 nM [^{3}H]AVP at various temperatures (4°C, 21°C, 30°C, and 37°C) for 10–30 min. Thereafter, binding was terminated by rinsing with ice-cold binding buffer to remove the unbound ligand and then the VSMCs were incubated for 10 min with 50 mM glycine in 150 mM NaCl (pH 3.0). Glycine washing at a pH of 3.0 or less has been shown to dissociate >95% ligand from its surface receptor (8). After this acid washing, the cells were rinsed with buffer to remove the unbound radioactive ligand. The count of internalized radioligand (acid resistant) was then measured after solubilizing the cells as described above. The

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Abbreviations: AVP, arginine vasopressin; VSMC, vascular smooth muscle cell; PAO, phenylarsine oxide.

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internalized [^{3}H]AVP is expressed as a percentage of total specific [^{3}H]AVP (intracellular [^{3}H]AVP plus acid released [^{3}H]AVP).

Loss of surface AVP receptors due to internalization. To quantify receptor internalization, confluent VSMCs were exposed to unlabeled 10 nM AVP at 4°C for 90 min or at 21°C for 30 min to promote AVP receptor internalization. Control experiments were performed with buffer alone. Thereafter, the incubation solution was removed, the cells were rinsed with ice-cold binding buffer, and then the cells were washed with acidic glycine to remove surface-bound AVP. [^{3}H]AVP (2 nM) binding studies were performed after washing the cells with binding buffer to restore extracellular pH to 7.4 at 4°C for 90 min.

Effect of phenylarsine oxide (PAO) on AVP receptor internalization. PAO is a covalent sulfhydryl modifying agent that inhibits ligand receptor internalization without depressing binding affinity or surface receptor density (10). VSMCs were pretreated with 0.1 mM PAO for 10 min before studying [^{3}H]AVP internalization at 21°C.

AVP Receptor Endosomal Processing. Previous studies have demonstrated that the acidic environment within endosomes is necessary for dissociation of several different ligands from their receptor (11, 12). To investigate whether the AVP–receptor complex requires endosomal processing, VSMCs were pretreated with either buffer or chloroquine (0.2 mM) for 30 min before exposing the cells to unlabeled 10 nM AVP for 30 min at 21°C to initiate internalization. Noninternalized AVP was then removed from the cells by rinsing with binding buffer and thereafter ice-cold acidic glycine washing was undertaken. The VSMCs were then incubated at 21°C for a further 30 min to allow the internalized AVP receptors to recycle to the cell surface. [^{3}H]AVP binding studies were then performed at 4°C to define whether raising intraendosomal pH with chloroquine limited the availability of AVP receptors for recycling to the cell surface. Binding in the presence of chloroquine is expressed as a percentage of binding after preexposure to buffer alone.

Kinetics of AVP Receptor Recycling by VSMCs. AVP receptor recycling was measured as the recovery of [^{3}H]AVP binding after prior AVP receptor internalization induced by preexposure to unlabeled 10 nM AVP at 21°C for 30 min. Unbound and surface-bound AVP was then removed by washing with binding buffer and thereafter with ice-cold acidic glycine before rewarming the cells to 21°C for various time periods to allow receptor recycling to occur.

Measurement of Intracellular-free Ca^{2+} Concentrations. VSMCs were loaded as described (8) with the cell-permeant acetoxymethylester form of the Ca^{2+}-sensitive fluorescent probe Fura 2-AM (2 μM) (Molecular Probes) for 30–45 min at 37°C and thereafter fluorescence (emission wavelength, 500 nm; excitation wavelengths, 340 and 380 nm) was measured at 37°C with a fluorescence spectrophotometer equipped with a thermostatically controlled cuvette holder (Perkin–Elmer 650-10S). Intracellular-free Ca^{2+} concentrations were calculated by the method of Grynkiewicz *et al.* (13).

Measurement of $^{45}Ca^{2+}$ Uptake After Receptor Cycling. VSMCs were equilibrated for 30 min in binding buffer with or without AVP at room temperature. AVP (10 nM) containing buffer induced receptor internalization. Thereafter, surface-bound AVP was removed by glycine washing. Before and after maximal receptor internalization and after receptor recycling to the surface, Ca^{2+} uptake measurements were performed. PSS (140 mM NaCl/4.6 mM KCl/1 mM $MgCl_2$/5 mM glucose/10 mM Hepes, pH 7.4) with or without 1 μM AVP containing 8 μCi of $^{45}Ca^{2+}$ per ml was added. After 5 min, the dish was placed on ice, the supernatant was aspirated, and cells were rinsed with Ca^{2+}-free PSS containing 2 mM EGTA. Thereafter, VSMCs were lysed and the cell-associated radioactivity was counted.

Statistical Analysis. The results are expressed as the means ± SEM. Unpaired Student's *t* test or analysis of variance (with Bonferroni correction) were used for statistical comparison. A P value of <0.05 was considered significant.

Materials. AVP and chloroquine were obtained from Sigma; $^{45}Ca^{2+}$ was purchased from Amersham; and [^{3}H]AVP was purchased from New England Nuclear.

RESULTS

AVP Binding to VSMCs. The binding of [^{3}H]AVP (2 nM) to cultured VSMC monolayers was saturable within 60 min at 4°C. Scatchard analysis of the binding data revealed a linear plot demonstrating a single class of AVP binding sites with a maximum number of binding sites (B_{max}) of 1.99 nmol per mg of cell protein and a dissociation constant (K_d) of 2.15 nM (Fig. 1). The binding of [^{3}H]AVP was displaced by coexposure to either excess unlabeled 10 μM AVP or a specific V_1 antagonist [d(CH_2)$_5$Tyr(Me)AVP] demonstrating that the single class of AVP receptors on VSMCs is of the V_1 variety (Fig. 2).

The addition of 1 μM AVP to VSMCs stimulated a significant increase in intracellular-free Ca^{2+} to a peak concentration of 479 ± 22 vs. 70 ± 5.2 nM ($P < 0.001$) in buffer-treated cells (Fig. 3). The presence of a specific V_1 antagonist (1 μM) for 3 min before addition of AVP (1 and 0.1 μM, respectively) completely abolished the Ca^{2+} mobilization responses previously demonstrated with AVP alone (Fig. 3).

AVP–Receptor Complex Internalization. At 4°C, regardless of the incubation time, all of the radiolabeled AVP added to VSMCs was released by washing the cells with the acidic glycine buffer and none was associated with solubilized cell protein (Fig. 4). This demonstrates that the acid washing procedure used in these studies was effective at displacing the surface-bound radioligand. Furthermore, it also demonstrates that AVP receptor internalization does not occur at 4°C and that binding studies performed at this temperature reflect cell-surface binding alone. With increasing temperature, total specific binding did not change significantly. When binding studies were performed at increased temperatures (21°C, 30°C, and 37°C), less radioactivity was recovered from the cell surface by acid glycine washing, and increasing quantities of radioactivity were associated with cell protein (Fig. 4). These findings indicate that internalization of the radioligand–receptor complex had occurred. Maximal translocation of [^{3}H]AVP to an intracellular compartment occurred after 20 min at 37°C or after 30 min at 30°C, indicating

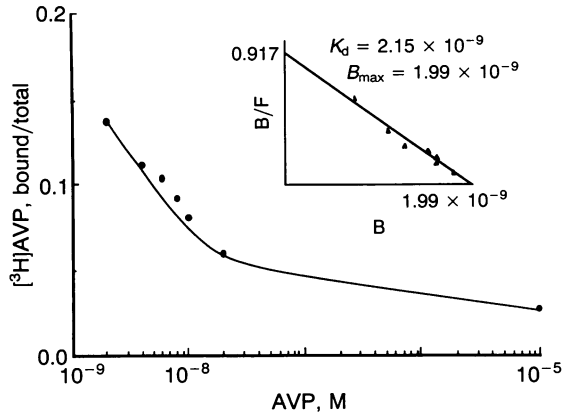

FIG. 1. Competition experiments show that increasing concentrations of unlabeled AVP displaced [^{3}H]AVP (2 nM) in VSMCs. Scatchard analysis revealed a linear plot demonstrating a single class of AVP binding sites (*Inset*). B, bound; F, free.

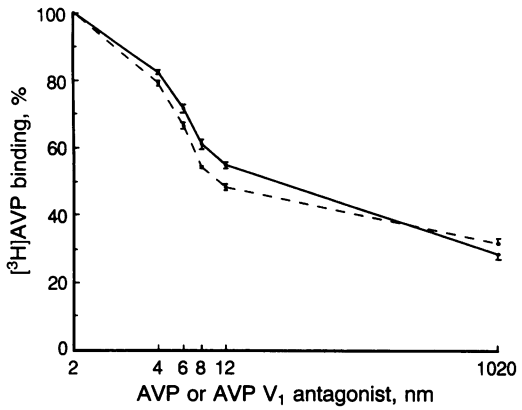

FIG. 2. The presence of unlabeled AVP (solid line) or a specific V_1 antagonist (dashed line) displaced bound [^{3}H]AVP to VSMCs in a similar and competitive manner.

that the internalization process is both time and temperature dependent. Maximal internalization of the radioligand occurred at 37°C and plateaued at 65% of bound radioactivity.

The next study investigated whether internalization of the AVP radioligand–receptor complex was paralleled by an equivalent loss of surface AVP receptors. After previous exposure to unlabeled 10 nM AVP at 4°C, no loss of [^{3}H]AVP surface binding sites occurred (buffer pretreatment, 100%; AVP pretreatment, 94.6 ± 1.3%), a finding compatible with an absence of ligand receptor internalization at this temperature. At 21°C, previous exposure to 10 nM AVP to induce receptor internalization resulted in a significant loss of [^{3}H]AVP binding sites (control, 100%; AVP, 71.2 ± 0.4%; $P < 0.0001$). This confirms that internalization of the AVP–receptor complex is associated with decreased availability of surface AVP binding sites.

To further confirm that the loss of surface AVP binding sites was due to ligand receptor internalization, binding studies were performed after preexposing VSMCs to PAO, an agent known to inhibit ligand receptor internalization without affecting ligand binding (10). The loss of [^{3}H]AVP binding sites measured after preexposure to 10 nM AVP to induce internalization at 21°C was effectively prevented by pretreatment of the VSMCs with 0.1 mM PAO (Fig. 5). However, the dose of PAO used in the present experiments did not affect ATP content or AVP-stimulated Ca^{2+} mobilization (14). Together, these studies demonstrate that AVP binding to its surface receptor on VSMCs results in internalization of the

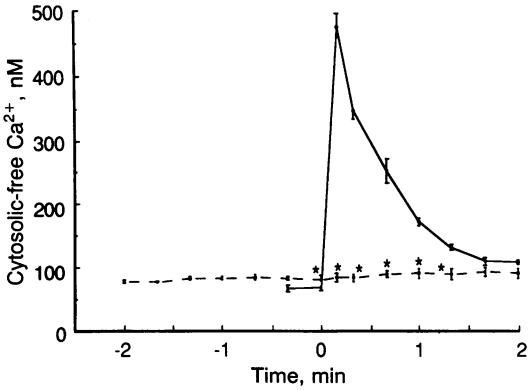

FIG. 3. AVP and V_1 antagonist effects on cytosolic-free Ca^{2+}. AVP (1 μM) stimulated increases in cytosolic-free Ca^{2+} in cultured VSMCs (solid line). The AVP effect was inhibited by pretreating the cells with a V_1 antagonist (1 μM) (dashed line). *, $P < 0.01$ vs. AVP alone.

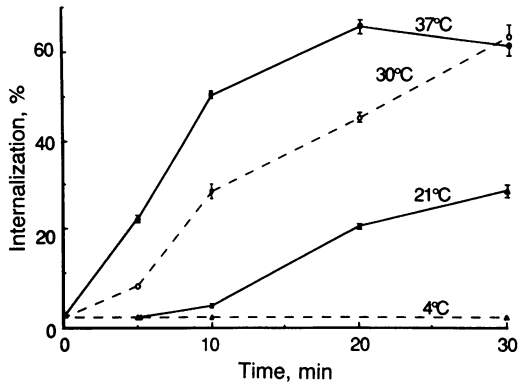

FIG. 4. [^{3}H]AVP–receptor complex internalization in VSMCs is time and temperature dependent. At 4°C, the AVP–receptor complex did not internalize. With increasing temperature and exposure time, the percentage of internalized ligand–receptor complexes progressively increased.

ligand–receptor complex in a time- and temperature-dependent manner.

AVP Receptor Endosomal Processing. The next study investigated whether, after internalization, the AVP–receptor complex requires processing within an endosomal compartment to allow ligand receptor dissociation. The mechanism of ligand receptor dissociation is strongly dependent on the acidic pH of endosomes (12). Endosomal pH can be elevated by pretreating VSMCs with a weak base (15). If endosomal processing is required to allow AVP receptor recycling, then chloroquine should depress the recycling mechanism. Chloroquine (0.2 mM) pretreatment of VSMCs did significantly depress AVP receptor recycling (Fig. 6) and thereby accentuated the 10 nM AVP-induced (30 min at 21°C) loss of cell-surface AVP receptors.

Kinetics of AVP Receptor Recycling. Internalization of the AVP receptor was induced by preexposing VSMCs to unlabeled 10 nM AVP at room temperature for 30 min. AVP pretreatment reduced AVP surface binding by 48.8% ± 0.17% compared to binding after treatment with buffer alone ($P < 0.001$). The recovery of these binding sites was, however, complete and rapid with a $t_{1/2}$ of <20 min at room temperature (Fig. 7). This rapid restoration of surface AVP receptors suggests that, after internalization, existing receptors are recycled without the requirement for new receptor synthesis. To further investigate this possibility, the recovery

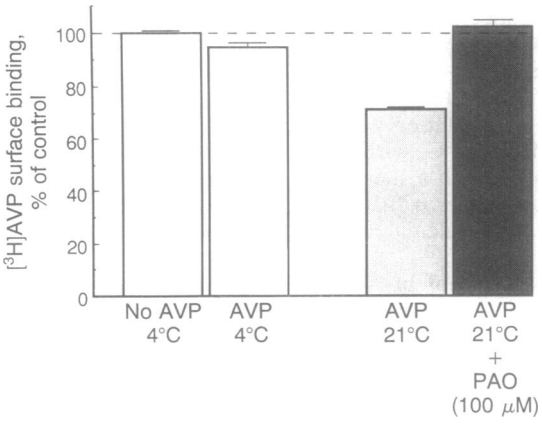

FIG. 5. Effect of temperature and PAO on the loss of AVP binding sites in VSMCs. Preexposure of VSMCs to unlabeled AVP (10 nM) induced (at 21°C) a loss of 28.8% surface binding sites ($P < 0.01$ vs. AVP at 4°C). A temperature of 4°C or pretreatment of VSMCs with PAO (100 μM) prevented AVP-induced internalization of the ligand–receptor complex.

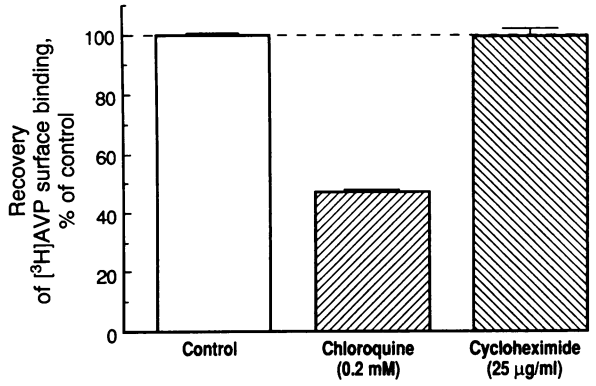

FIG. 6. Effects of chloroquine and cycloheximide on AVP–receptor recovery after AVP (10 nM)-induced (21°C; 30 min) receptor internalization in VSMCs. Chloroquine (0.2 mM), which enhances endosomal pH, significantly inhibited ($P < 0.01$ vs. control) the recovery of [^{3}H]AVP surface binding sites. Cycloheximide (25 μg/ml), a blocker of protein synthesis, did not alter the recovery of AVP binding sites in VSMCs.

of surface AVP receptors was determined after preexposing VSMCs to cycloheximide, a protein synthesis inhibitor. Cycloheximide (25 μg/ml) had no effect on the recovery of AVP binding sites (buffer pretreatment, 100% binding; cycloheximide pretreatment, 99.8% ± 2.2% binding) (Fig. 6), suggesting that receptor recycling is solely responsible for the immediate recovery of the AVP receptor after AVP receptor internalization by VSMCs.

Determining the Function of the AVP Receptor After Binding, Internalization, and Recycling. Before receptor internalization, 1 μM AVP stimulated Ca^{2+} uptake from 100% (basal) to 122.5% ± 3.4% ($P < 0.01$). After exposure to 10 nM AVP, internalization of receptors occurred and there was a consequent loss of 31.5% surface AVP receptors. This loss in surface receptors was paralleled by a significant attenuation of the Ca^{2+} uptake response to rechallenge with 1 μM AVP (before internalization, 122.5% ± 3.4%; after internalization, 101.8% ± 2.8%; $P < 0.01$). Time was then allowed for recycling to occur to replenish surface AVP receptors. Further exposure to 1 μM AVP at this point resulted in a normal Ca^{2+} uptake response (AVP response before internalization, 122.5% ± 3.4%; AVP response after internalization and recycling, 128.4% ± 4.8%; not significant) (Fig. 8). These results demonstrate that changes in the magnitude of the intracellular signal response to AVP are temporally related to the availability of AVP surface receptors throughout the

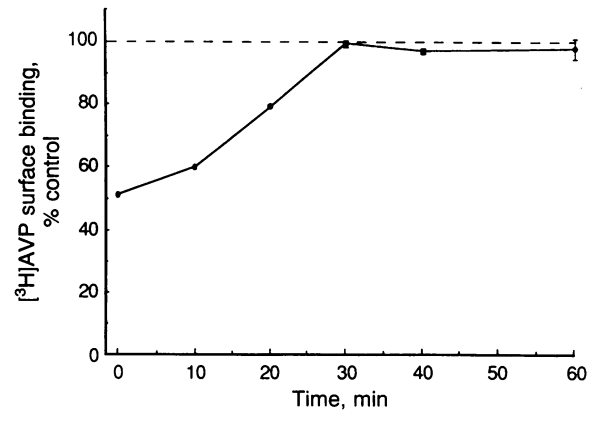

FIG. 7. Time course of AVP–receptor recycling after internalization. AVP (10 nM)-induced internalization of 49% of ligand–receptor complexes in VSMCs. At room temperature, the recovery of these binding sites occurred with a $t_{1/2}$ of <20 min.

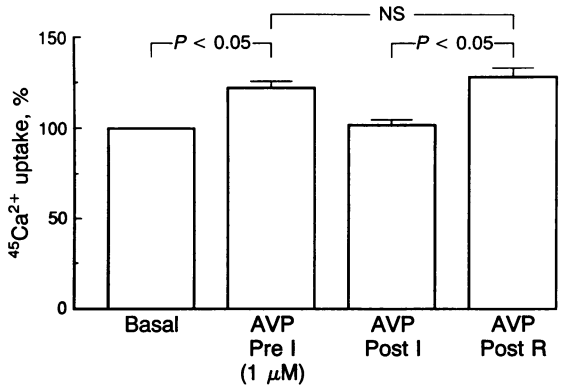

FIG. 8. AVP-stimulated Ca^{2+} uptake relates to the availability of AVP surface receptors. $^{45}Ca^{2+}$ uptake by VSMCs in response to AVP was measured before AVP–receptor internalization (Pre I), upon rechallenge with AVP, postreceptor internalization (Post I), or upon further rechallenge with AVP, postreceptor recycling (Post R). NS, not significant.

internalization and recycling process in VSMCs. Furthermore, these results specifically demonstrate that the V_1 receptor functions normally and immediately after recycling to the surface of VSMCs.

DISCUSSION

The present study demonstrates that a single class of AVP receptors on the surface of cultured VSMCs and AVP initiates intracellular signal transduction via this receptor. Displacement studies demonstrated that either unlabeled AVP or a specific V_1 antagonist displaces [^{3}H]AVP bound to VSMCs. These results confirm the presence of a V_1 AVP receptor on VSMCs in culture. In accordance with this conclusion, AVP-mediated signal transduction, measured as AVP-induced increases in cytosolic-free Ca^{2+} and Ca^{2+} efflux (data not shown), was also prevented by pretreating VSMCs with the specific V_1 AVP antagonist.

The B_{max} (1.99 nmol per mg of protein) and K_d (2.15 nM) for the V_1 receptor on VSMCs as defined in the present study are similar to that reported for the V_1 receptor in hepatocytes (16). It is of note, however, that in hepatocytes, the B_{max} showed considerable variation, with the highest occurring in freshly isolated hepatocytes but declining within a few hours. In contrast, in VSMCs the B_{max} for the AVP receptor was stable and reproducible for >10 cell passages.

In spite of the similarities in binding kinetics for the V_1 receptor in both VSMCs and hepatocytes, there are marked differences in the time required for postbinding events, including recycling of the V_1 receptor in these different cell types. In VSMCs, internalization and recycling of the V_1 receptor is rapid, with a $t_{1/2}$ of 20 min at 21°C for internalization and a $t_{1/2}$ of 20 min for recycling. In hepatic tissue at 18°C, however, V_1 internalization (3–6 min) is even faster (16).

Although the present study in cultured rat VSMCs demonstrates that the V_1 receptor recycles after binding AVP, in another study in which cultured A10 cells, a smooth muscle cell line, were used AVP receptor recycling was not shown (7). A significant difference in the latter study is that a fluorescent AVP probe was used. Although this method is sensitive and allows the ligand–receptor complex to be localized within the cell, the results are at best qualitative. It also seems likely that the fluorescent AVP dissociates from the receptor after internalization, thus preventing further tracking of the receptor through the recycling process. In addition, fluorescent labeling of AVP makes the hormone more lipophilic and modifies the kinetics of its binding to

surface receptors. It is therefore conceivable that the fluorescent labeling of the AVP molecule may also modify its intracellular processing. Finally, it is unknown whether phenotypic changes in the A10 cell line could modify the processing of receptors in these cells when compared to freshly cultured VSMCs. Whatever the explanation for the failure to demonstrate AVP receptor recycling in A10 cells, our results demonstrate that the V_1 receptor does recycle in VSMCs.

It is of interest that receptors for other vasoconstrictor peptide hormones, notably endothelin (3) and angiotensin II (10), have similar internalization and recycling pathways and similar time courses for intracellular ligand–receptor trafficking in VSMCs as reported for the vasoconstrictor V_1 receptor in the present study. As each of these vasoconstrictors elicits actions in vascular tissue via similar postreceptor signal transduction mechanisms, it raises the possibility that intracellular processing of these different receptors may not be regulated by the receptor itself but by some common factor in the postreceptor signaling mechanism. AVP, endothelin, and angiotensin II each activates protein kinase C in VSMCs, and recent studies have suggested that activation of protein kinases C may play an important role in regulating surface-receptor density (8, 17).

Before receptor recycling, the ligand may dissociate from the receptor. Studies of the insulin receptor suggest that although ligand–receptor dissociation may not be a prerequisite for recycling to occur, the cycling process is considerably prolonged without dissociation (18). Ligand–receptor dissociation generally occurs within the acidic environment of the prelysosomal endosomes located in the peripheral cytoplasm. Weak bases such as chloroquine can raise the pH within the endosome and thus inhibit ligand–receptor dissociation (19), an effect that might delay or prevent recycling (15). The results of the present study demonstrate that chloroquine pretreatment of VSMCs does inhibit recycling of the V_1 receptor after its internalization, thus suggesting that endosomal processing of the V_1 AVP–receptor complex is an important component of the normal recycling mechanism. If, in the present study, a sequestered pool of subplasmalemmal receptors, rather than recycled receptors, accounted for the return to the cell surface of biologically active receptors, it is difficult to understand how such an effect would be blocked by chloroquine. In the chloroquine studies, the receptors were internalized; thus, in this setting it could be argued that any sequestered subplasmalemmal pool of receptors should have been mobilized to the cell surface. While some nonendosomal effect of chloroquine might have occurred, the most likely explanation of the effect of chloroquine is that endosomal processing is necessary for V_1 receptor recycling.

The rapidity of receptor recycling in VSMCs suggests that *de novo* receptor synthesis is not required. This conclusion was supported by the observation in the present study that cycloheximide, in a dose sufficient to inhibit protein synthesis and therefore new receptor generation (16), had no effect on the AVP recycling mechanism. Prevention of AVP receptor internalization by PAO did not result in a significant change in surface binding sites. These findings, however, do not completely exclude the possibility that receptor synthesis is a continuous low grade process required to replenish the AVP receptor pool and/or that variations in the rate of receptor synthesis may play an important role in the more long-term regulation of the receptor B_{max}.

Finally, the results of the present study indicate that the receptor recycling mechanism may be of considerable physiologic significance. The rapid internalization of the V_1 AVP–receptor complex by VSMCs desensitizes the VSMCs to immediate rechallenge with AVP. This effect occurred in association with a significant decrease in the availability of AVP surface receptors. This decrease in receptor number was paralleled by a decrease in AVP-induced intracellular signaling, thus supporting previous observations of AVP-induced homologous desensitization in VSMCs (8). The close relationship between the magnitude of loss of surface receptors and the attenuation of signaling after V_1 receptor internalization also suggests that there are few, if any, spare V_1 receptors on VSMCs. A similar conclusion was recently reached for the angiotensin II receptor in rat mesenteric VSMCs (10).

Another physiologic implication of these studies is that recovery from homologous desensitization to rechallenge by AVP is dependent, at least in part, on the time required to recycle the V_1 receptor. In vascular tissue, where AVP leads to vasoconstriction and thus plays an important role in regulation of systemic blood pressure and regional tissue perfusion, it would seem appropriate that the surface receptor density be replenished by virtue of a rapid receptor recycling time.

Although these results suggest a potential role for receptor recycling in the hormone desensitization mechanism, it is important to define whether the recycled receptor retains its biologic function. In the present study in VSMCs, intracellular signaling in response to AVP correlated with the availability of AVP surface receptors throughout the receptor internalization and recycling mechanism. In addition, the recycled receptor was shown to be immediately functional in terms of its capacity to initiate intracellular signaling on rechallenge with AVP.

In conclusion, the present study demonstrates that AVP binds to a single class of V_1 receptors on VSMCs. After binding, the ligand–receptor complex is rapidly internalized in a time- and temperature-dependent manner. It has been demonstrated here that the acidic environment of intracellular endosomes is necessary to allow the ligand to dissociate from the receptor and that the free receptor is then recycled rapidly to the cell surface, where it is immediately functional. This study also suggests that the recycling process is an important determinant of the immediate AVP homologous desensitization mechanism in VSMCs.

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1. Schrier, R. W. (1988) *N. Engl. J. Med.* **319** (Parts 1 and 2), 1065–1072, 1127–1134.
2. Nabika, T., Velletri, P. A., Lovenberg, W. & Beaven, M. A. (1985) *J. Biol. Chem.* **260**, 4661–4670.
3. Resink, T. J., Scott-Burden, T., Boulanger, C., Weber, E. & Bühler, F. R. (1990) *Mol. Pharmacol.* **38**, 244–252.
4. Abrahamson, D. R. & Rodewald, R. (1981) *J. Cell. Biol.* **91**, 270–280.
5. Carpenter, G. & Cohen, S. (1976) *J. Cell Biol.* **71**, 159–171.
6. Mostov, K. E. & Blobel, G. (1982) *J. Biol. Chem.* **257**, 11816–11821.
7. Lutz, W., Sanders, M., Salisbury, J. & Kumar, R. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6507–6511.
8. Caramelo, C., Tsai, P., Okada, K., Briner, V. & Schrier, R. W. (1991) *Am. J. Physiol.* **29**, F46–F52.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
10. Ullian, M. E. & Linas, S. L. (1989) *J. Clin. Invest.* **84**, 840–846.
11. Haigler, H. T., Maxfield, F. R., Willingham, M. C. & Pastan, I. (1980) *J. Biol. Chem.* **255**, 1239–1241.
12. Ohkuma, S. & Poole, B. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3327–3331.
13. Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450.
14. Tsai, P., Williams, B. & Schrier, R. W. (1991) *J. Am. Soc. Nephrol.* **2**, 466 (abstr.).
15. Maxfield, F. R. (1982) *J. Cell Biol.* **95**, 676–681.
16. Fishman, J. B., Dickey, B. F., Bucher, N. L. R. & Fine, R. E. (1985) *J. Biol. Chem.* **260**, 12641–12646.
17. Eldar, H., Zisman, Y., Ulrich, A. & Livneh, E. (1990) *J. Biol. Chem.* **265**, 13290–13296.
18. Carpentier, J.-L., Gazzano, H., van Obberghen, E., Fehlmann, M., Freychet, P. & Orci, L. (1986) *J. Cell Biol.* **102**, 989–996.
19. Dean, R. T., Jessup, W. & Roberts, C. R. (1984) *Biochem. J.* **217**, 27–40.