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In vitro desensitization of isolated nephron segments to vasopressin

(kidney tubules/microdissection/thick ascending limb/glucagon/calcitonin)

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ABSTRACT **Recent studies have demonstrated that *in vivo* administration of 1-deamino-8-D-arginine-vasopression, an analog of arginine-8-vasopressin, induces homologous desensitization to vasopressin in the thick ascending limb of the loop of Henle. Desensitization has been documented by a decreased physiological response to vasopressin *in vivo* and by a reduced cAMP accumulation in the cortical thick ascending limb (CTAL). By measuring cAMP content in single isolated medullary thick ascending limbs (MTALs), we now report that desensitization can occur all along the thick ascending limb and, more importantly, that it can also be induced *in vitro*. In a first series of experiments, we observed that 1 hr after *in vivo* injection of 1-deamino-8-D-arginine-vasopressin, MTALs were desensitized by 80% to vasopressin, whereas the effects of the other hormones acting on the same cyclase pool (glucagon, calcitonin) were fully maintained. In a second set of experiments, desensitization was induced *in vitro* by vasopressin, the natural hormone. A 60-min preincubation of MTALs with vasopressin caused a marked (up to 86%) and highly reproducible desensitization. The process was dose and time dependent. The apparent K_a for desensitization was 0.2 nM, and the half-maximal effect was obtained within 20 min. The desensitization induced *in vitro* by vasopressin was again essentially homologous in nature, with 80% of the maximal stimulation of cAMP accumulation being obtained in the presence of glucagon. Desensitization to vasopressin was observed in the presence and absence of indomethacin, indicating that it is independent of prostaglandin synthesis. It is concluded that (*i*) vasopressin and its analog 1-deamino-8-D-arginine-vasopressin cause marked desensitization in the CTAL and MTAL and (*ii*) the low vasopressin concentrations required to induce desensitization and the rapid onset of the process suggest that it has a physiological significance.**

Desensitization, the process by which cell exposure to a hormone reduces the biological response to subsequent stimulation, is known to occur in various tissues and has been shown to affect several signaling pathways (1). The best characterized of these pathways, the adenylate cyclase system, can undergo two main types of desensitization depending on the site at which the alterations occur (1, 2). The nonselective type (i.e., heterologous desensitization) is characterized by a decrease of cell responsiveness to all agents acting through the same transducing pathway. A more selective process referred to as homologous desensitization is observed when the decreased cell responsiveness only concerns the hormone responsible for the desensitization process. This kind of desensitization is initiated by selective alterations at the receptor level—namely, by phosphorylation reactions (2).

In mammals, the antidiuretic hormone vasopressin reduces the urinary excretion rate of water and solutes by its action on several nephron segments. In the collecting duct, it increases the reabsorption of water, NaCl (3, 4), and urea (5). In the thick ascending limb, it increases the reabsorption of Na (6), Cl (6, 7), Mg (6), and Ca (6). Although current evidence indicates that cAMP is the physiological second messenger mediating vasopressin effects in both the thick ascending limb (7–9) and the collecting duct (3, 5), the desensitization process does not operate similarly in the two segments. In a previous study (10), we found that *in vivo* administration of 1-deamino-8-D-arginine-vasopressin (dDAVP), an analog of antidiuretic hormone selective for receptors coupled to adenylate cyclase, caused marked desensitization to vasopressin in the cortical thick ascending limb (CTAL) but not in the cortical collecting duct. Thus, the thick ascending limb appears to be a site of preferential desensitization to vasopressin in the kidney. Desensitization of the CTAL was of the homologous type and altered the generation of cAMP (10) and the biological response (11).

With the previous approach, based on *in vivo* dDAVP injections, it could not be demonstrated that desensitization indeed comes from a direct effect of vasopressin on its target cells nor could the hormone concentrations and time required to induce the process be determined accurately. Accordingly, in the present study, we developed a completely *in vitro* approach, depending on cAMP accumulation measurements in single isolated nephron segments. These experiments were performed on the MTAL in order to extend to another portion of this nephron segment the observations made with the *in vivo* desensitization procedure on the CTAL.

MATERIALS AND METHODS

Methods. The experiments were made on intact MTALs obtained by microdissection from collagenase-treated kidneys of male Sprague–Dawley rats (N = 29). The methods used for obtaining and processing the nephron segments, adapted from those of Chabardes *et al.* (12), have been described elsewhere (10) and will only be recalled briefly.

Composition of Solutions. The microdissection solution contained the following (in mM): 137 NaCl, 5 KCl, 0.8 $MgSO_4$, 0.34 Na_2HPO_4, 0.44 KH_2PO_4, 1 $MgCl_2$, 1 $CaCl_2$, 4 $NaHCO_3$, 10 sodium acetate, 5 glucose, and 20 Hepes (pH 7.5). It also contained 1 mg of bovine serum albumin per ml (fraction V; Miles). This solution was used for microdissection and preincubation of nephron segments.

The incubation solution had the same composition as the microdissection solution except for the presence of 1 mM IBMX and 1 mg of bacitracin per ml. Since prostaglandins are known to inhibit hormone-dependent cAMP accumulation in the rat MTAL (13, §), in some experiments indomethacin

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Abbreviations: AVP, arginine-8-vasopressin (antidiuretic hormone); dDAVP, 1-deamino-8-D-arginine-vasopressin; IBMX, 3-isobutyl-1-methylxanthine; MTAL and CTAL, medullary and cortical parts of the thick ascending limb, respectively.

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§Chabardes, D., Montegut, M. & Siaume-Perez, S., in Proceedings of the 10th International Congress on Nephrology, July 26–31, 1987, London, p. 249 (abstr.).

(final concentration, 5 μM) was added to all media (i.e., microdissection and incubation solutions) to avoid possible inhibitory effects of endogenous prostaglandins on cAMP synthesis (15).

Except where indicated, all chemicals were purchased from Sigma.

Isolation of Nephron Segments. Laboratory chow of constant composition and tap water were provided to all rats until the experiments began. The left kidney was prepared for microdissection as described (10). MTALs were dissected from the inner stripe of the outer medulla. Isolated nephron segments were transferred with 1 or 2 μl (see below) of microdissection solution to a concave bacteriological glass slide. The slide was immediately covered with a second vaseline-coated glass slide and kept on ice until preincubation. One to three nephron fragments (total length, 0.4–2.2 mm, depending on the experimental conditions) were transferred to each slide to obtain an adequate length for accurate cAMP determination.

Desensitization by *in Vivo* Administration of dDAVP. The object of these experiments was to determine whether *in vivo* administration of high doses of dDAVP induced, in the MTAL, a reduction of vasopressin-dependent cAMP accumulation, as previously demonstrated in the CTAL (10). Accordingly, as in our previous studies, desensitization was elicited by i.m. injections of dDAVP (2 μg/day for 3 days). These experiments were carried out in the presence of indomethacin, as in the previous CTAL studies.

The tubular samples, transferred with 2 μl of microdissection solution, were preincubated for 10 min at 30°C by immersing the sealed glass slide in a water bath. Immediately after preincubation, incubation was initiated by adding 2 μl of incubation solution containing 2 mM IBMX and the hormone to be tested at twice the concentration required for the final 4-μl incubation volume. The final concentration of IBMX was thus 1 mM. The incubation, performed at 35°C by again immersing the glass slide in a water bath, lasted 4 min. It was stopped by transferring the sample by pipette onto a 10-μl droplet of formic acid in absolute ethanol (5%, vol/vol) in a polypropylene tube. After overnight evaporation to dryness at 40°C, 50 μl of 50 mM potassium phosphate buffer (pH 6.2) was added to the dry extracts and the samples were kept frozen at −24°C until determination of cAMP content.

These experiments were performed on five control (i.e., untreated) and five desensitized rats studied 1 hr after the last injection of dDAVP. In addition to the effects of AVP, those of glucagon (purified porcine glucagon; Novo Industries, Bagsvaerd, Denmark) and of synthetic human calcitonin (Cibacalcin; Ciba–Geigy, Basel) were always examined.

***In Vitro* Desensitization of the MTAL to Vasopressin.** Isolated segments were transferred with 1 μl of microdissection solution to a glass slide. The preincubation period, carried out at 30°C, was initiated by adding either 1 μl of microdissection medium (control samples) or 1 μl of the same medium containing AVP (desensitized samples). IBMX was omitted during preincubation. That the absence of IBMX indeed avoided the accumulation of cAMP was checked by measuring the cAMP content of samples preincubated with 0–10^{-8} M AVP: these were always found to be close to, or even below, the detection threshold of the assay—i.e., <3 fmol/mm (n = 37 samples from four experiments). As an example, the actual amount of cAMP accumulating during a 60-min preincubation with 10^{-8} M AVP in the absence of IBMX ranged between <3 and 6.4 fmol/mm (mean value: 3.4 fmol/mm·60 min, n = 7). The incubation itself (4 min at 35°C) was performed as indicated above by adding, immediately after preincubation, 2 μl of incubation medium containing IBMX and the tested hormone(s). Because of the low amount of cAMP accumulated during preincubation, the results are presented as fmol/mm·4 min of incubation time.

The total duration of preincubation was always 60 min. Thus, in experiments in which the time course of desensitization was investigated, the preincubation was initiated without AVP and then completed after hormone addition when required.

Measurement of cAMP. cAMP was measured on acetylated samples using an enzyme immunoassay, for which the characteristics and application to tubular segments have been recently described (10). The calibration curve ranged from 4 to 500 fmol per tube and enabled amounts of cAMP between 5 and 130 fmol per tube to be measured accurately. Half displacement of the enzymatic tracer was usually obtained for cAMP contents of 20–25 fmol per tube.

Calculations. The amount of cAMP present in each sample was expressed in fmol/mm of tubular length per 4 min of incubation time (fmol/mm·4 min^{-1}). Each experiment consisted of several replicate samples (mean ± SD = 7 ± 2) for each experimental condition. Results are presented as means ± SEM of N experiments, with the individual mean value from one rat (or experiment) as a single datum. Results of representative experiments are given as means of n replicate samples for each condition studied. Statistical comparisons between groups were performed using variance analysis or Student's paired t test, as appropriate. A P value of 0.05 or less was considered significant.

RESULTS

Desensitization of the Rat MTAL by *in Vivo* Injections of dDAVP. Fig. 1 shows the effects of dDAVP injection 1 hr before kidney removal on subsequent hormone-induced cAMP accumulation *in vitro*. In control rats, cAMP accumulation was of similar magnitude in the presence of either AVP, glucagon, or calcitonin, all tested at concentrations high enough to ensure maximal effects (10). In dDAVP-injected rats, AVP-dependent cAMP accumulation was markedly lower than in control rats, whereas the effects of glucagon and calcitonin were fully maintained. These results therefore provide evidence of an 80% homologous desensitization of MTAL to AVP.

***In Vitro* Desensitization of the Rat MTAL to AVP.** We first investigated whether prolonged *in vitro* preincubation of nephron segments compromised the ability of the cells to synthesize cAMP. Accordingly, a dose–response curve for AVP-dependent cAMP accumulation was established. As

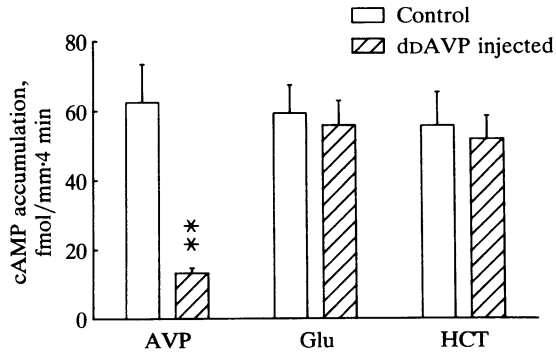

FIG. 1. Desensitization by *in vivo* injections of dDAVP. Each column is the mean value ± SEM of five experiments. Desensitization was induced by i.m. injections of dDAVP (2 μg/day for 3 days). The last injection was performed 1 hr before kidney removal. Hormone concentrations in the assay medium were as follows: vasopressin (AVP), 10^{-8} M; glucagon (Glu), 10^{-8} M; human calcitonin (HCT), 10^{-7} M. Basal values, measured after a preincubation period of 10 min in the absence of added hormone and IBMX, were <3 fmol/mm in both groups. Asterisks indicate a significant difference compared with values obtained with AVP in control rats (P < 0.01, variance analysis).

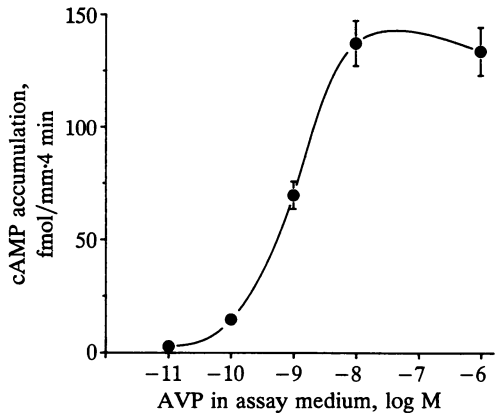

FIG. 2. Dose-dependent effect of vasopressin on cAMP accumulation in the MTAL. Each point is the mean value ± SEM of 9–17 replicate samples obtained in two different experiments performed in the presence of indomethacin. Basal values, measured in the absence of added hormone in the preincubation and incubation medium, were <2 fmol/mm and are therefore not indicated.

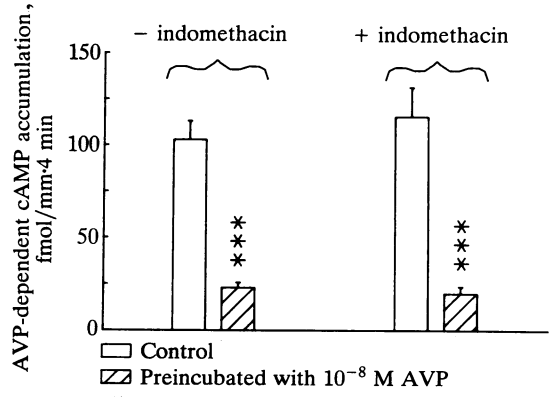

FIG. 3. *In vitro* desensitization of the MTAL to vasopressin. Each column is the mean value ± SEM of six experiments. Isolated MTALs were preincubated for 60 min in the absence (control) or presence (hatched columns) of 10^{-8} M AVP but without IBMX. A 4-min incubation was then carried out in the presence of 1 mM IBMX and 10^{-8} M AVP in all samples. Asterisks indicate a significant difference between control segments and those preincubated in the presence of vasopressin ($P < 0.001$, paired t test). The percentage of desensitization to vasopressin averaged 83% ± 1% and 78% ± 6% in the presence and absence of indomethacin, respectively.

shown in Fig. 2, a marked (≈50-fold) increase of cAMP accumulation was observed when the AVP concentration was raised from 10^{-11} to 10^{-8} M. The apparent K_a for vasopressin-dependent cAMP accumulation (≈1 nM) was similar to the one obtained for a shorter, 10-min preincubation period (ref. 10 and unpublished observations). The maximal amount of cAMP accumulated, however, was higher in the former case than in the latter (compare Fig. 1 and Fig. 2). Thus, prolonged preincubation in a hormone-free medium potentiated AVP-dependent cAMP production. For this observation to be substantiated we compared, in the same experiment, the effects of a maximal concentration of AVP (10 nM) on samples preincubated either 10 or 60 min at 30°C. The highest response was always obtained in MTAL preincubated for 60 min: AVP-dependent cAMP accumulation (fmol/mm·4 min) was 62.1 ± 6.7 and 117.4 ± 12.3 in samples preincubated 10 and 60 min, respectively ($P < 0.001$, $N = 7$). The higher amount of cAMP measured in samples that had undergone a prolonged preincubation was not due to cAMP accumulated during preincubation (in which IBMX was absent) since no cAMP could be detected at the end of this period (i.e., accumulation rate, <3 fmol/mm·60 min; see *Materials and Methods*). In view of the increase of maximal AVP-induced cAMP accumulation observed after prolonged preincubation in the hormone-free medium and to avoid time-related effects in subsequent experiments, all samples were preincubated for 60 min, irrespective of the duration of exposure to AVP.

In vitro preincubation of MTAL with 10^{-8} M AVP led to a marked reduction of AVP-dependent cAMP accumulation (Fig. 3). Desensitization of MTAL was seen in all experiments and ranged from 71.5% to 86.5% (81.0% ± 2.0%, $N = 12$). As shown in Fig. 3, the process was observed in the presence and absence of indomethacin, suggesting that it was not related to prostaglandin synthesis.

The desensitization induced *in vitro* by AVP was dose and time dependent (Figs. 4 and 5). Fig. 4 indicates that the threshold for desensitization was close to 0.1 nM and that the maximal effect was obtained for 1–10 nM. This concentration range closely resembles that at which adenylate cyclase is stimulated by AVP (compare Fig. 2 and Fig. 4).

The time course of desensitization is illustrated in Fig. 5. For these studies, preincubation was performed in the presence of 10^{-9} M AVP, since this concentration had been found to induce a nearly maximal desensitization. The kinetic experiments indicated that there were three distinct phases in the desensitization process. (*i*) A latent period of about 10

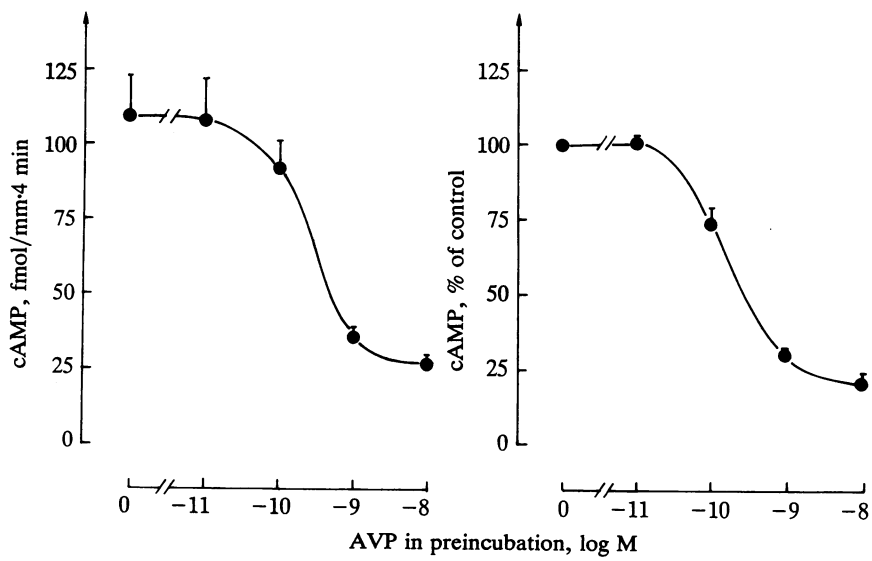

FIG. 4. Dose-dependent desensitization of the MTAL to vasopressin. Isolated MTALs were preincubated for 60 min in the presence of the indicated hormone concentrations. A 4-min incubation was then carried out in the presence of 1 mM IBMX and 10^{-8} M AVP. (*Left*) Results from a representative experiment with absolute amounts of cAMP measured under each condition (each point is the mean ± SEM of five or six replicate samples). (*Right*) Mean values of five similar experiments (except that preincubation with 10^{-11} M AVP was made in only three of these experiments). Data of each experiment were expressed as % of their respective controls. The mean value for cAMP accumulation in control segments was 113.6 ± 16.6 fmol/mm·4 min ($N = 5$). Half-maximal desensitization was obtained for an AVP concentration of 2×10^{-10} M.

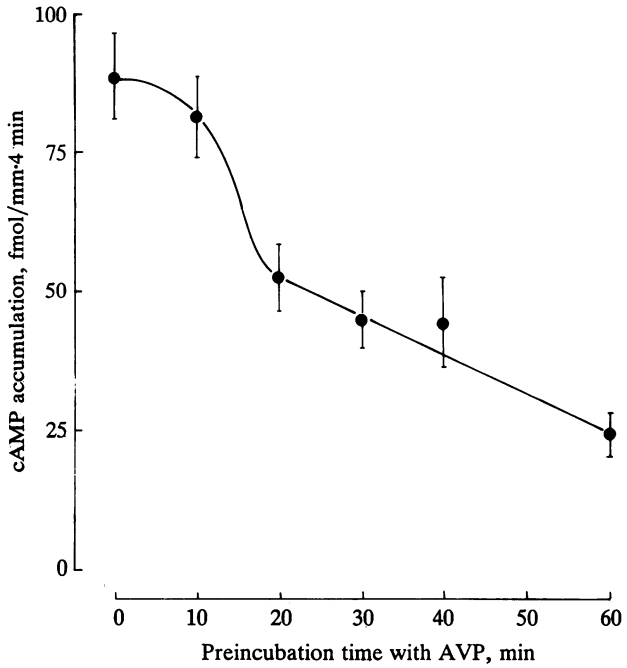

FIG. 5. Kinetics of vasopressin-induced desensitization in the MTAL. Isolated MTALs were preincubated in the presence or absence of 10^{-9} M AVP. Time on the abscissa indicates duration of exposure to AVP immediately before the 4-min incubation period, with the overall preincubation period lasting 60 min in all cases. After completion of preincubation, a 4-min incubation was carried out in the presence of 1 mM IBMX and 10^{-8} M AVP to initiate cAMP accumulation. Each point is the mean value ± SEM of three experiments. Significant desensitization was obtained for preincubation times with AVP ≥ 20 min (P at least <0.01, variance analysis).

min during which no desensitization occurred. (*ii*) A short phase of rapid desensitization followed, so that by 20 min desensitization had already reached 56% ± 1% of its maximum (i.e., of that obtained after 60 min of preincubation). (*iii*) Between 20 and 60 min there was a sustained slower decrease.

The specificity of desensitization induced by *in vitro* incubation with AVP was investigated by measuring the effects of glucagon on cAMP accumulation (Fig. 6). Because of the low

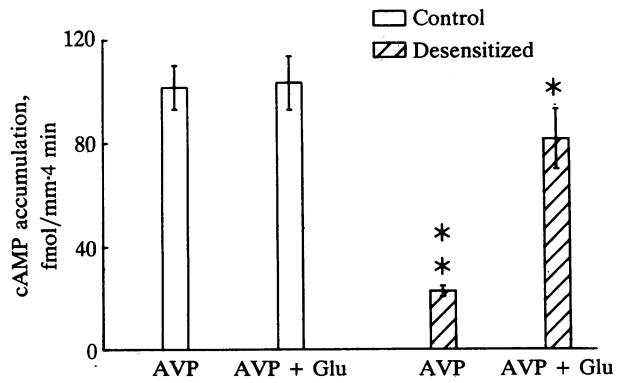

FIG. 6. Comparative effects of AVP alone and of AVP and glucagon (Glu) in control MTALs and MTALs desensitized to AVP. Isolated MTALs were preincubated for 60 min in the presence or absence of 10^{-8} M AVP and then incubated for 4 min with 1 mM IBMX and AVP alone or incubated with AVP and glucagon (AVP + Glu). Hormone concentrations in the assay medium were 10^{-8} M for AVP and glucagon. Each column is the mean value ± SEM of seven experiments. **, Significantly different from the three other groups ($P < 0.01$); *, significantly different from the values obtained with AVP and glucagon in control segments ($P < 0.05$). Statistical comparisons among groups were performed using variance analysis.

amount of material obtained by the microdissection technique and the potential damage to tubular cells by rinses and transfers into fresh solutions, the effects of glucagon alone could not be determined in segments preincubated with AVP. Both hormones were therefore present together during the 4-min incubation time. Under control conditions, AVP alone already induced maximal cAMP accumulation: when glucagon was also present, no further increase was noted, confirming that the two hormones act on the same cyclase pool (8). In MTALs preincubated with AVP, the AVP-dependent cAMP accumulation was only 22.6% ± 1.6% of that observed in controls. However, AVP-desensitized MTALs were still capable of producing large amounts of cAMP since in the presence of additional glucagon a nearly maximal stimulation was obtained. The value obtained in the presence of AVP and glucagon was nevertheless slightly lower in desensitized than in control segments ($P < 0.05$), indicating that a small heterologous desensitization might also be present. However, desensitization was clearly a receptor-mediated process. Thus, a 60-min preincubation with forskolin (16 μM) only induced a 7.7% ± 5.5% reduction of the ability of this agent to stimulate cAMP production, whereas in the same experiments preincubation with 10 nM AVP led to the most currently observed desensitization to hormone action of 83% ± 1% (n = 16 and 15 samples for forskolin and AVP, respectively; means of two experiments). This indicates that a prolonged (i.e., 60 min) stimulation of adenylate cyclase is not sufficient by itself to reduce the ability of the cells to synthesize cAMP.

DISCUSSION

Previous studies from this laboratory had demonstrated that *in vivo* administration of dDAVP induced a marked reduction of AVP-dependent cAMP accumulation in the CTAL (10). Desensitization of CTAL was selective for AVP, with the effects of glucagon and calcitonin on cAMP synthesis being unaltered. The present work, which shows that a similar *in vivo* treatment with dDAVP also markedly desensitizes the MTAL to vasopressin, extends our previous observations. The extent of desensitization is closely similar in the two segments. A 75% desensitization was reported for CTAL when the experiment was performed 2 hr after dDAVP injection and, in the present study, 1 hr after administering dDAVP there was an 80% desensitization of MTAL to AVP. Thus, the process of desensitization would appear to operate similarly in CTAL and MTAL. A desensitization of both segments may thus have been responsible for the reduced biological effects of AVP in the superficial loop of Henle of similarly dDAVP-treated rats (11).

Although the preceding series of experiments clearly demonstrated a homologous form of desensitization, definitive evidence that the process was induced by a direct interaction of the agonist with its target cells was still lacking. The *in vitro* approach on isolated nephron segments clearly helps to define the characteristics of desensitization. (*i*) We can rule out the intervention of several factors that may have been altered after hormone administration, such as disturbance in body fluid homeostasis, increase in blood pressure, or changes in concentration of unrelated hormones in blood. (*ii*) With this approach we were able to show that AVP, the natural antidiuretic hormone in rats, can itself induce desensitization. (*iii*) We have demonstrated that desensitization is a dose-dependent phenomenon with a threshold as low as that of adenylate cyclase activation. (*iv*) It was possible to study the kinetics of desensitization.

Studies of desensitization of the adenylate cyclase pathway in several cell systems have shown that the process is dose dependent. The physiological significance of desensitization is still often questioned, however, since high hormone con-

centrations (in the micromolar range) are generally needed to induce the phenomenon. This has been observed for several hormones, including vasopressin, when desensitization was evaluated by measuring hormone-sensitive adenylate cyclase activity (16–18). In experiments carried out on the pig kidney, preincubation with dDAVP of membranes obtained from the whole medulla caused desensitization of adenylate cyclase when the peptide concentration exceeded 10^{-7} M (16). Subsequent experiments performed on an established cell line from the pig kidney (17) or on primary cultures of the rabbit collecting duct (18) revealed a poor ability of these cells to be desensitized by vasopressin. Preincubation of intact cells with vasopressin led to a dose-dependent desensitization of adenylate cyclase activity, but here again maximum desensitization required a hormone concentration of 10^{-6} M (17, 18). It should be stressed, however, that such cells are poorly responsive to vasopressin, with the maximal activation of adenylate cyclase also occurring at 10^{-6} M (17, 18). By measuring cAMP accumulation in intact, freshly isolated cells, we were able to show that low hormone concentrations can desensitize the thick ascending limb. The AVP concentration in normal rat blood is $\approx 2 \times 10^{-12}$ M and may increase to $2–3 \times 10^{-11}$ M during dehydration. A further increase to $3–5 \times 10^{-10}$ M is observed during stressful stimuli such as hypotensive hemorrhage (19). The K_a for desensitization (2×10^{-10} M) found in the present study lies in this range. Even more striking is the fact that the thresholds for desensitization and hormone-induced cAMP accumulation were found to be similar. This indicates that if the action of AVP in the thick ascending limb is of physiological significance, as is likely (20, 21), desensitization may be a physiological mode of regulation of vasopressin action in this nephron segment.

The specificity of desensitization deserves comment. Although the effect of glucagon was largely preserved, this hormone did not fully restore maximal cAMP accumulation when desensitization was induced *in vitro*. This suggests that a small (≈20%) heterologous desensitization was also present in these experiments. The occurrence of homologous and heterologous desensitization mechanisms in a single cell system has been described for the β-adrenergic receptor (22–24). However, since the greater part of desensitization is clearly selective for AVP, it is safe to conclude that desensitization in the MTAL is essentially homologous in nature. This is also supported by the data obtained after *in vivo* injection of dDAVP. In this case, the effects of glucagon and calcitonin were fully maintained.

The potentiation of AVP-dependent cAMP production by prolonged preincubation without hormone is interesting. It was not observed in medullary collecting tubules studied under similar conditions (unpublished observations), indicating it exhibits some segmental specificity. Takaichi and Kurokawa (14) also reported an increase of AVP-dependent cAMP production in the mouse MTAL after prolonged preincubation, but in their studies this phenomenon only occurred in the presence of pertussis toxin. Here again the medullary collecting tubule was not affected by such a treatment. Since pertussis toxin suppressed the inhibitory effect of high ambient Ca concentration on AVP-dependent cAMP accumulation in MTAL, the authors suggested that the 1.8 mM Ca present in the control condition could be sufficient to partially inhibit AVP action (14). In our experiments, the Ca concentration was only 1 mM. Thus, it is possible that owing to this lower Ca concentration, the potentiating effect of prolonged incubation was observed even in the absence of pertussis toxin.

In conclusion, the present study demonstrates the feasibility of investigating desensitization to peptide hormones at the level of single nephron segments. In the thick ascending limb, we observed that AVP induces a marked and rapid desensitization that may be of physiological relevance. The multiple hormonal control of adenylate cyclase in the thick ascending limb (8) may have thus even more physiological importance than previously suspected (20). It could help to maintain a functional response in cells particularly apt to become desensitized to vasopressin.

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