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## Multiple Cytosolic Calcium Signals and Membrane Electrical Events Evoked in Single Arginine Vasopressin-stimulated Corticotrophs\*

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The action of arginine vasopressin (AVP) on cytosolic free  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) was investigated in single rat pituitary corticotrophs using indo-1 microfluorimetry, in part in combination with the monitoring of membrane electrical events with the perforated patch-clamp technique. In corticotrophs showing the series of short-lived  $[Ca^{2+}]_i$  rises (transient pattern) in response to corticotropin-releasing factor, 100 nM AVP evoked either the transient pattern or a  $[Ca^{2+}]_i$  spike followed by a sustained plateau (spike/plateau pattern). Not all corticotrophs responded to changes in AVP concentration in the same manner. Some cells exhibited a concentration-dependent increase in  $[Ca^{2+}]_i$ ; transient activity, whereas others showing the spike/plateau at high AVP concentrations responded to low agonist concentrations by two  $[Ca^{2+}]_i$  responses: a slow rising step or two to three sinusoidal-like oscillations. Combined  $[Ca^{2+}]_i$  and patch-clamp recordings as well as manipulation of extracellular  $Ca^{2+}$  showed that both transient pattern and the plateau of spike/plateau response depended on  $Ca^{2+}$  entry mainly through voltage-gated, dihydropyridine-sensitive  $Ca^{2+}$  channels. By contrast, step, oscillations, and spike were due to  $Ca^{2+}$  release from internal stores. These  $Ca^{2+}$ -mobilizing responses caused the activation of  $Ca^{2+}$ -activated, apamin-sensitive  $K^+$  channels, which led to a membrane hyperpolarization. These results reveal cell-specific  $[Ca^{2+}]_i$  signals and associated electrical events in individual AVP-stimulated corticotrophs.

An increase in cytosolic free  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ )<sup>1</sup> in endocrine cells regulates several cell functions, including

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<sup>1</sup> The abbreviations used are:  $[Ca^{2+}]_i$ , cytosolic free calcium concentration; ACTH, adrenocorticotropic hormone; CRF, corticotropin-releasing factor; AVP, arginine vasopressin; indo-1/AM, indo-1 pentaacetoxymethyl ester; TEA, tetraethylammonium chloride; TTX, tetrodotoxin; BAPTA/AM, bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid, tetra(acetomethyl) ester; GnRH, gonadotropin-releasing hormone; Ins-1,4,5- $P_3$ , inositol 1,4,5-trisphosphate.

secretion (1, 2), gene expression (3-6), and cell proliferation (6). The mechanisms by which an increase in  $[Ca^{2+}]_i$  regulates one and not another of these  $Ca^{2+}$ -dependent functions remain unclear. One possibility would be that a single agonist regulates different  $Ca^{2+}$ -dependent functions by generating distinct  $[Ca^{2+}]_i$  signals under different conditions (7). Single cell studies have recently revealed that endocrine cells could encode the agonist concentration by generating  $[Ca^{2+}]_i$  responses that differ with regard to their pattern and source of  $Ca^{2+}$  (7-9). Another possibility would be that a given cell type comprises several subsets, each with its characteristic agonist-stimulated  $[Ca^{2+}]_i$  signal (10, 11).

Release of adrenocorticotropic hormone (ACTH) and other pro-opiomelanocortin-derived peptides from corticotrophs in the anterior pituitary gland is  $Ca^{2+}$ -dependent (12-14) and has two major secretagogues, corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) (15-17). The action of CRF on  $[Ca^{2+}]_i$  is well characterized. Single cell  $[Ca^{2+}]_i$  measurements have demonstrated that most if not all, corticotrophs show a single mode of  $[Ca^{2+}]_i$  response to CRF: a series of short-lived rises in  $[Ca^{2+}]_i$ , or transient, due to  $Ca^{2+}$  entry through voltage-gated  $Ca^{2+}$  channels (18). As reported in other endocrine cells (19-23), these  $[Ca^{2+}]_i$  transients are characterized by their kinetics: a time to peak <1 s and a decaying phase lasting several seconds (18). The effects of CRF on  $Ca^{2+}$  entry and related  $Ca^{2+}$ -dependent functions are thought to be mediated via the generation of cyclic AMP and activation of the cyclic AMP-dependent protein kinase, protein kinase A (24-27). By contrast, the action of AVP on  $[Ca^{2+}]_i$  remains puzzling. In the pituitary, AVP seems to act through a single class of receptors (V1b receptor) (28, 29), which would be coupled to cyclic AMP-independent pathways (25, 30-36). Patch-clamp experiments carried out on human corticotrophs have revealed that AVP can enhance  $Ca^{2+}$  entry by directly acting on voltage-gated  $Ca^{2+}$  channels (37). Single cell  $[Ca^{2+}]_i$  recordings performed on normal rat corticotrophs have recently shown that AVP at high concentrations (100 nM to 1  $\mu$ M) can produce a large, rapid, and transient increase in  $[Ca^{2+}]_i$  ("spike") assumed to be due to  $Ca^{2+}$  release from  $Ca^{2+}$ -sequestering stores (38, 39). The spike is followed by an elevated  $[Ca^{2+}]_i$  plateau dependent on extracellular  $Ca^{2+}$  (38). These observations suggest that corticotrophs are responsive to two agonists, which generate distinct  $[Ca^{2+}]_i$  signals, and that at least AVP may produce more than one signal.

We investigated the diversity and the nature of AVP-induced  $[Ca^{2+}]_i$  signals in normal rat corticotrophs. Since corticotrophs present spontaneous  $[Ca^{2+}]_i$  transients linked to voltage-gated  $Ca^{2+}$  entry (18),  $[Ca^{2+}]_i$  measurements with the  $Ca^{2+}$  fluorescent probe indo-1 were combined with the patch-clamp monitoring of plasma membrane electrical events (membrane potential or macroscopic ionic currents).

## MATERIALS AND METHODS

**Cell Culture**—Male rat (Sprague-Dawley) pituitary glands were enzymatically dissociated as previously described (40). The dispersed cells were then plated on polyornithine-treated glass coverslips and cultured 2–5 days in Ham's F-10 medium supplemented with 10% fetal calf serum in a humidified 95% air-5%  $CO_2$  atmosphere at 37 °C. Single corticotrophs were morphologically and immunocytochemically identified as described previously (18). Furthermore, in some identified cells (see Fig. 1), the responsiveness for AVP was compared with the  $[Ca^{2+}]_i$  responses to CRF known to evoke characteristic responses (18).

**Measurements of Cytosolic Free  $Ca^{2+}$  Concentration and Membrane Electrical Events**—Before each electrophysiological and/or microfluorimetric experiment, the culture medium was replaced by a modified Hank's solution containing 142.6 mM NaCl, 5.6 mM KCl, 2 mM  $CaCl_2$ , 0.8 mM  $MgCl_2$ , 5 mM glucose, 10 mM HEPES, and buffered to pH 7.3 with NaOH. The cells were loaded with the fluorescent  $Ca^{2+}$  probe indo-1 by exposure to 5  $\mu$ M indo-1 pentaacetoxymethyl ester (indo-1/AM, Calbiochem, Paris, France) and 0.02% (w/v) pluronic F-127 (Molecular Probes, Eugene, OR) at room temperature for 30 min (20).  $[Ca^{2+}]_i$  was estimated from indo-1 fluorescence by the ratio method using single wavelength excitation (355 nm) and dual wavelength emission (405 and 480 nm) recorded with two separate photometers (Nikon-France, Paris, France) (20).

$[Ca^{2+}]_i$  levels were calculated on-line (lag time < 10  $\mu$ s) by using an analog circuit that converted the ratio of emission fluorescence ( $F_{405}/F_{480}$ ) to  $Ca^{2+}$  concentration according to the formula derived by Grynkiewicz *et al.* (9, 20, 41). Microfluorimetric experiments were performed at 37 °C. When microfluorimetric recordings of  $[Ca^{2+}]_i$  were combined with electrophysiological recordings of membrane electrical events (membrane potential or macroscopic ionic current), the perforated whole-cell patch-clamp technique was used (18, 21, 23, 42). This technique consists in perforating the patch of membrane under the recording electrode with monovalent ion selective pores (diameter of about 0.8 nm) formed by the polyene antibiotic nystatin (Sigma) present in the electrode solution. These pores provide a low resistance electrical connection between the patch pipette solution and the cytosol without the loss of diffusible intracellular compounds involved in the response to stimuli (18, 23, 42). The electrode solution contained: 10 mM KCl, 10 mM NaCl, 70 mM  $K_2SO_4$ , 7 mM  $MgCl_2$ , and 5 mM HEPES (pH 7.3); nystatin (100  $\mu$ g/ml) was added to the electrode solution before filling patch pipettes. Perforation was usually achieved within 5–10 min after seal formation (access resistance < 50 megohms). Electrophysiological recordings were carried out using the Bio-Logic RK 300 (Bio-Logic, Claix, France) patch-clamp amplifier equipped with a 100-megohm feedback resistor in the head stage. Linear  $[Ca^{2+}]_i$  and patch-clamp signals were simultaneously displayed on a pen recorder (model 2200S, Gould, Paris, France), and fed into separate channels of an analog to digital converter (TL-1/DMA Interface, Axon Instrument, Inc., Foster City, CA), digitized, stored, and analyzed on an IBM PS/2 model 30 personal computer using pCLAMP software (version 5.5.1, Axon).

**Test Substances**—Solutions containing agonists or antagonists were pressure-ejected from an extracellular micropipette (tip diameter, 2–5  $\mu$ m), the tip of which was positioned within 10–20  $\mu$ m of the recorded cell. The concentrations reported are those in the pressure pipette. AVP and ovine CRF were purchased from UCB-Bioproducts (Braine l'Alleud, Belgium). To obtain  $Ca^{2+}$ -free solution,  $CaCl_2$  was omitted from, and 2 mM EGTA was added to, modified Hank's solution. The  $Ca^{2+}$  channel blocker cadmium chloride ( $Cd^{2+}$ ),  $K^+$  channel blockers apamin, tetraethylammonium chloride (TEA), and *d*-tubocurarine, as well as  $Na^+$  channel blocker tetrodotoxin (TTX) were purchased from Sigma. The dihydropyridine L-type  $Ca^{2+}$  channel blocker PN 200-110 was kindly provided by Sandoz-France (Paris, France). The  $Ca^{2+}$  ionophore ionomycin and BAPTA/AM were purchased from Calbiochem. When cells were preloaded with BAPTA, indo-1-loaded cells were exposed to 50  $\mu$ M BAPTA/AM in  $Ca^{2+}$ -free solution for 30 min at room temperature, washed twice, and then recorded in  $Ca^{2+}$ -free solution (9).

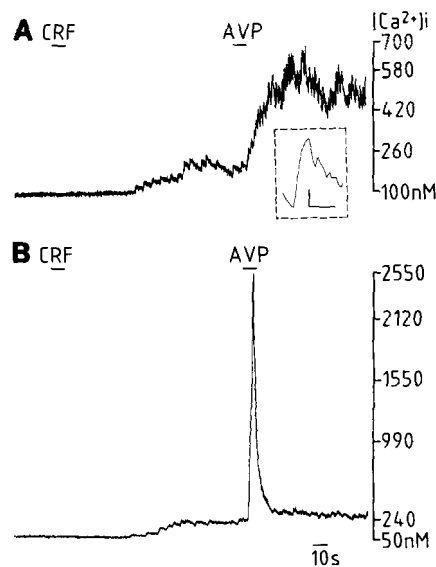
**Statistics**—Results are expressed as mean  $\pm$  standard deviation. Paired and un-paired Student's *t* test and Mann-Whitney *U* tests were used when appropriate. Differences with  $p < 0.05$  were considered significant.

## RESULTS

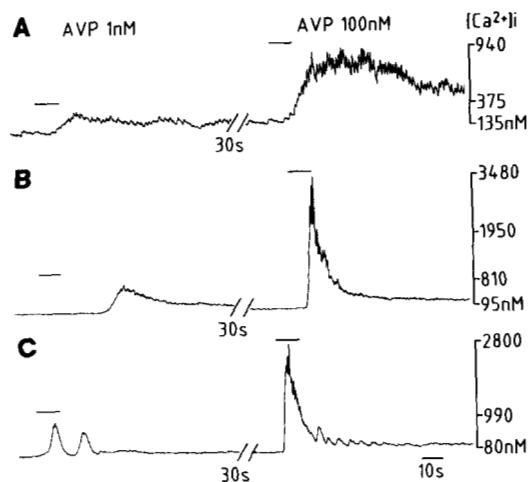
**Evidence for Two Major  $[Ca^{2+}]_i$  Response Patterns to AVP in Individual CRF-responsive Corticotrophs**—Pituitary corti-

cotrophs, in response to CRF, present a characteristic and reproducible pattern of  $[Ca^{2+}]_i$  variations consisting of a series of low amplitude  $[Ca^{2+}]_i$  transients (18). In corticotrophs that responded to 100 nM CRF in this fashion, 100 nM AVP elicited two distinct patterns of  $[Ca^{2+}]_i$  variations (Fig. 1). In 32 out of 67 cells (48%), AVP increased the frequency of CRF-induced  $[Ca^{2+}]_i$  transients ( $0.31 \pm 0.22$  and  $0.44 \pm 0.23$  Hz prior to and after AVP application, respectively;  $p < 0.05$ ) (Fig. 1A), which had a time to peak < 1 s (Fig. 1A, inset), while in the remainder (52%), AVP caused after a short delay a  $[Ca^{2+}]_i$  spike that was often followed by a plateau (Fig. 1B). The increase in  $[Ca^{2+}]_i$  transient frequency led to a summation of  $[Ca^{2+}]_i$  transients, which resulted in a prolonged elevation of  $[Ca^{2+}]_i$  above 500 nM (Fig. 1A). The spike response consisted of a rapid rise of  $[Ca^{2+}]_i$  to the micromolar range, followed by a slow decay to a plateau which was higher than the pre-AVP level (Fig. 1B).

**Concentration Dependence of the AVP-induced  $[Ca^{2+}]_i$  Responses in Corticotrophs**—To determine if the two patterns of  $[Ca^{2+}]_i$  response to AVP were concentration-dependent and required prior exposure to CRF, we challenged the same cell to 1 and 100 nM AVP (Fig. 2). Consistent responses were observed at AVP concentrations  $\geq 1$  nM. Three distinct patterns of  $[Ca^{2+}]_i$  increase were observed at 1 nM AVP: low amplitude, short-lived transients with a fast rising phase (time to peak < 1s) (Fig. 2A, 41 of 79 cells, 52%); delayed step, which was prolonged low amplitude transient increase with a slow rising phase (time to peak > 2 s) (Fig. 2B, 36%); and two to three sinusoidal-like oscillations exhibiting roughly equal rising and decaying rates (Fig. 2C, 12%). In all cases, the amplitude of the response was larger at 100 nM AVP. Most cells that presented  $[Ca^{2+}]_i$  transients at 1 nM also presented transients at 100 nM (Fig. 2A). The frequency but not the amplitude of  $[Ca^{2+}]_i$  transients increased upon raising AVP concentration from 1 to 100 nM (frequency:  $0.19 \pm 0.09$  versus



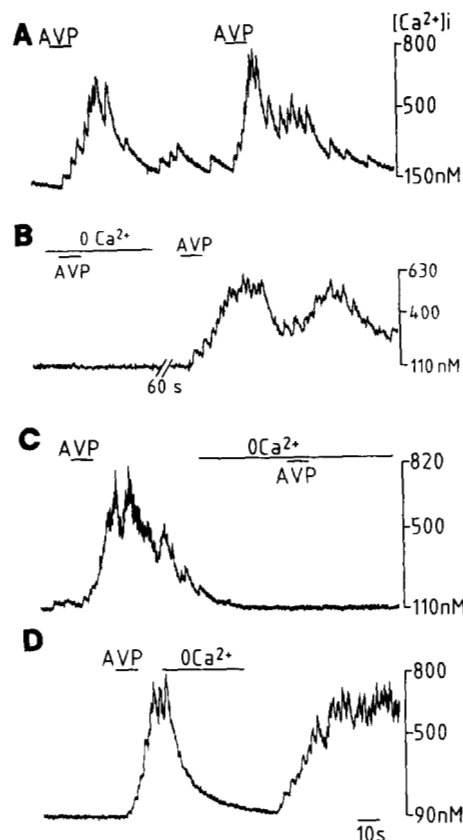
**FIG. 1. Heterogeneity of  $[Ca^{2+}]_i$  responses to AVP (100 nM) obtained in single CRF-responsive corticotrophs.**  $[Ca^{2+}]_i$  was measured in single indo-1-loaded corticotrophs that were sequentially stimulated with 100 nM CRF and 100 nM AVP. Agonists were applied to single cells from different micropipettes placed in their vicinity during the time indicated by horizontal bars. A, typical AVP-induced  $[Ca^{2+}]_i$  transient pattern in a CRF-responsive cell. The inset shows the profile at higher resolution of a  $[Ca^{2+}]_i$  transient during AVP stimulation. Vertical and horizontal scale bars correspond to 25 nM and 800 ms, respectively. B, typical AVP-induced  $[Ca^{2+}]_i$  spike/plateau pattern in another CRF-responsive cell.



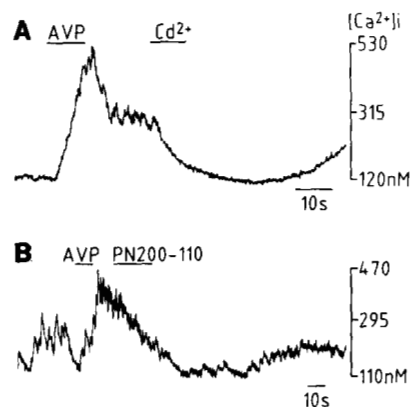
**FIG. 2. Concentration dependence of AVP-induced  $[Ca^{2+}]_i$  responses.** Each trace represents separate corticotrophs that were sequentially challenged by AVP at 1 and 100 nM, respectively. *A*, 1 and 100 nM AVP produced  $[Ca^{2+}]_i$  transients that show an increase in frequency upon raising peptide concentration. *B*, 1 nM AVP produced a slow rising phase, low amplitude  $[Ca^{2+}]_i$  step, and 100 nM AVP caused a rapid high amplitude spike followed by a plateau. *C*, 1 nM AVP produced sinusoidal  $[Ca^{2+}]_i$  oscillations, and 100 nM AVP caused a rapid high amplitude spike followed by an oscillating plateau.

$0.35 \pm 0.15$  Hz,  $p < 0.05$ ; amplitude:  $239 \pm 96$  nM versus  $373 \pm 237$  nM,  $p > 0.05$ ). Cells that presented either a step or oscillations at 1 nM AVP responded to 100 nM by a rapid spike (Fig. 2, *B* and *C*). The spike was followed by a steady or oscillating plateau in cells that responded to 1 nM AVP with a step or oscillations, respectively. Cells that presented either single steps or oscillations at 1 nM never presented a high frequency transient activity at 100 nM. Only 2 out of 41 cells presented a transition from the transient to the spike pattern upon increasing AVP concentration (not shown). Therefore, the pattern of the AVP responses was not dependent on prior CRF exposure and a given pattern seems to be set by the individual cell, while the amplitude of the response is concentration-dependent.

**$[Ca^{2+}]_i$  Transient Response Pattern Is Due to  $Ca^{2+}$  Entry during Action Potential Firing**—The kinetic features of  $[Ca^{2+}]_i$  transients evoked by AVP resembled those due to voltage-gated  $Ca^{2+}$  entry in spontaneously active and CRF-stimulated corticotrophs (18). Therefore, we tested whether AVP-stimulated transient pattern depended on  $Ca^{2+}$  entry. To this end, the action of AVP was investigated while extracellular  $Ca^{2+}$  was manipulated. In the presence of normal extracellular  $Ca^{2+}$ , successive applications of 100 nM AVP separated by 1 min or more generated reproducible series of  $[Ca^{2+}]_i$  transients (Fig. 3*A*). The removal and chelation of external  $Ca^{2+}$  with 2 mM EGTA prevented the  $[Ca^{2+}]_i$  response to either a first (Fig. 3*B*) or a second (Fig. 3*C*) AVP application. Furthermore, the application of  $Ca^{2+}$ -free solution during the AVP response curtailed the transient activity and  $[Ca^{2+}]_i$  dropped to the prestimulation level.  $[Ca^{2+}]_i$  transient activity quickly reappeared when extracellular  $Ca^{2+}$  was restored (Fig. 3*D*). Similar results were observed with  $Cd^{2+}$  (200  $\mu$ M,  $n = 25$ ), an inorganic blocker of both L-type and T-type voltage-gated  $Ca^{2+}$  channels (Fig. 4*A*). When the dihydropyridine L-type  $Ca^{2+}$  channel blocker PN 200-110 was applied at 100 nM during the AVP response, the peak amplitude of  $[Ca^{2+}]_i$  transients was markedly (60–90%) reduced ( $n = 23$ ). This effect was partially reversible within 1–5 min after termination of the drug application (Fig. 4*B*). Thus, it seems likely that the  $[Ca^{2+}]_i$  transient pattern evoked by AVP depends on  $Ca^{2+}$



**FIG. 3. Effect of extracellular  $Ca^{2+}$  removal on the AVP-induced  $[Ca^{2+}]_i$  transient pattern.** Each  $[Ca^{2+}]_i$  recording represents separate corticotrophs, which were challenged twice with 100 nM AVP as indicated by the appropriate horizontal bar. *A*, the cell responded to two identical AVP challenges by producing  $[Ca^{2+}]_i$  transients. *B*, the removal of extracellular  $Ca^{2+}$  as indicated by the horizontal bar blocked the  $[Ca^{2+}]_i$  transients produced by a first AVP challenge. *C*, the removal of extracellular  $Ca^{2+}$  as indicated by the horizontal bar blocked the  $[Ca^{2+}]_i$  transients produced by the second AVP challenge. *D*, the removal of extracellular  $Ca^{2+}$  during the AVP response curtailed the  $[Ca^{2+}]_i$  transients.



**FIG. 4. Effects of  $Ca^{2+}$  channel blockers on the  $[Ca^{2+}]_i$  transient pattern induced by AVP.** Each  $[Ca^{2+}]_i$  recording was made in separate cells.  $Ca^{2+}$  channel blockers were applied in the vicinity of cells in which 100 nM AVP had evoked  $[Ca^{2+}]_i$  transients. *A*, 200  $\mu$ M  $Cd^{2+}$  reversibly abolished  $[Ca^{2+}]_i$  transients. *B*, 100 nM PN 200-110 induced a prolonged decrease of the peak amplitude of  $[Ca^{2+}]_i$  transients.

entry via voltage-gated  $Ca^{2+}$  channels.

We examined whether voltage-gated  $Na^+$  channels may participate in the establishment of  $[Ca^{2+}]_i$  transients triggered upon AVP stimulation. Application of the  $Na^+$  channel

blocker TTX ( $10 \mu M$ ) caused a reversible block of  $[Ca^{2+}]_i$  transients in a third of corticotrophs (8 out of 24 cells) (Fig. 5).

To further investigate the involvement of voltage-gated  $Ca^{2+}$  entry in AVP-induced  $[Ca^{2+}]_i$  transients,  $[Ca^{2+}]_i$  monitoring was combined simultaneously with perforated patch-clamp membrane potential recording. Fig. 6A shows with an extended time scale a spontaneous action potential (lower panel) and its associated  $[Ca^{2+}]_i$  transient (upper panel). Application of  $100 \text{ nM}$  AVP slightly depolarized (about  $5 \text{ mV}$ ) the cell, caused low amplitude fluctuations of the membrane potential, and increased the action potential frequency ( $0.10 \pm 0.18$  and  $0.19 \pm 0.20 \text{ Hz}$  before and after AVP application, respectively,  $n = 6$ ,  $p < 0.05$ ) without any marked change in the action potential shape (Fig. 6B). The increase in the frequency of  $[Ca^{2+}]_i$  transients paralleled the increase in action potential firing rate, indicating that the increase in maximum  $[Ca^{2+}]_i$  was not due to an increase in action potential amplitude or duration, but rather to the increased action potential frequency.

Taken together, these data provide evidence that corticotrophs showing a  $[Ca^{2+}]_i$  transient pattern upon AVP stimulation employ voltage-gated  $Ca^{2+}$  entry during action potential firing to raise their  $[Ca^{2+}]_i$ .

**AVP-triggered Step, Oscillation, and Spike  $[Ca^{2+}]_i$  Patterns Are Due to  $Ca^{2+}$  Release from Intracellular  $Ca^{2+}$  Sequestering Stores**—The step, oscillation, and spike  $[Ca^{2+}]_i$  patterns differ in terms of kinetics from the transient pattern. However, these  $[Ca^{2+}]_i$  patterns resembled those due to  $Ca^{2+}$  release from internal stores (8). To determine whether  $Ca^{2+}$  entry was involved in these patterns, the effects of AVP were investigated while extracellular  $Ca^{2+}$  was manipulated. The removal and chelation of extracellular  $Ca^{2+}$  with  $2 \text{ mM}$  EGTA prior to  $1 \text{ nM}$  AVP stimulation was ineffective in altering the  $1 \text{ nM}$  AVP-triggered step patterns (mean peak amplitude:  $410 \pm 225 \text{ nM}$  ( $n = 11$ ) versus  $375 \pm 183 \text{ nM}$  ( $n = 6$ ) for control versus  $Ca^{2+}$ -deprived cells;  $p > 0.05$ ) (Fig. 7A). Cells producing  $[Ca^{2+}]_i$  oscillations in response to  $10 \text{ nM}$  AVP were also insensitive to removal of external  $Ca^{2+}$  (mean peak value:

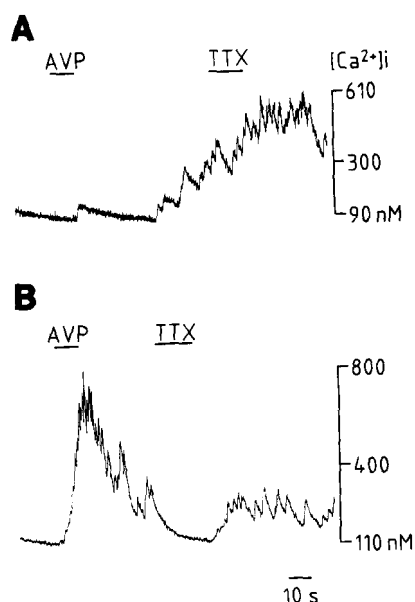


FIG. 5. Effects of the  $Na^+$  channel blocker TTX on the  $[Ca^{2+}]_i$  transient pattern induced by AVP. TTX ( $10 \mu M$ ) was applied in the vicinity of cells in which  $100 \text{ nM}$  AVP had evoked  $[Ca^{2+}]_i$  transients. A, TTX had no detectable effect on the AVP-evoked response. B, TTX reversibly abolished  $[Ca^{2+}]_i$  transients.

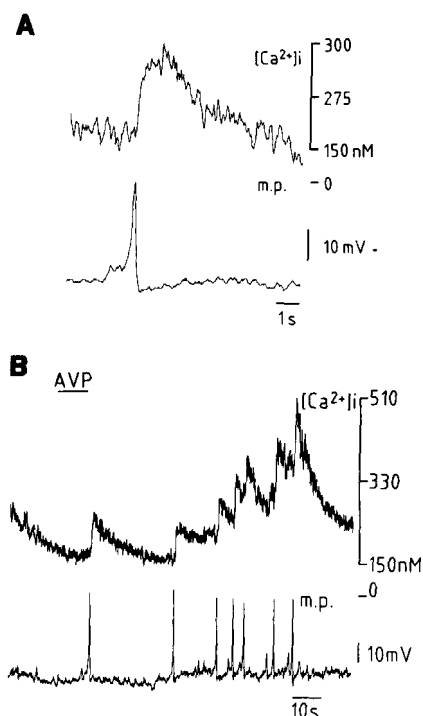
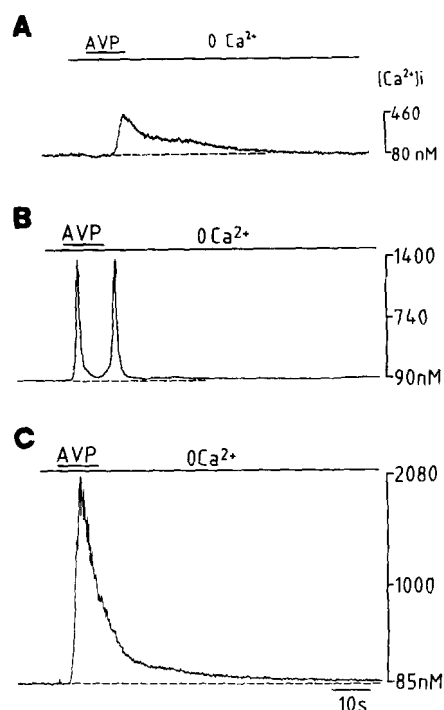


FIG. 6. Simultaneous recording of the membrane potential and  $[Ca^{2+}]_i$  in an AVP-stimulated corticotroph showing a  $[Ca^{2+}]_i$  transient pattern. Combined recordings of  $[Ca^{2+}]_i$  (upper panel) and membrane potential (lower panel) were obtained by combining  $Ca^{2+}$  microfluorimetry with the patch-clamp technique in its perforated whole-cell configuration as detailed under "Materials and Methods." The membrane potential was recorded under current-clamp (zero current) conditions. A, a spontaneous action potential and its associated  $[Ca^{2+}]_i$  transient recorded prior to AVP stimulation. B, AVP was applied at  $100 \text{ nM}$  as indicated.

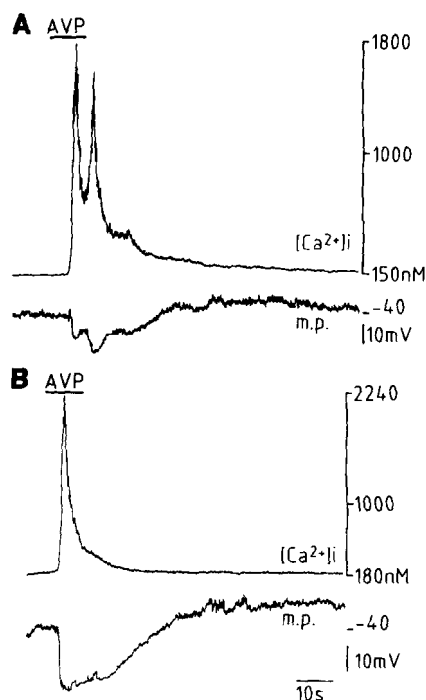
$1496 \pm 746 \text{ nM}$  ( $n = 14$ ) versus  $1773 \pm 2266 \text{ nM}$  ( $n = 11$ ) for control versus  $Ca^{2+}$ -deprived cells;  $p > 0.05$ ) (Fig. 7B). Similarly, the  $[Ca^{2+}]_i$  spike evoked by  $10$ – $100 \text{ nM}$  AVP was unaffected by short term removal of external  $Ca^{2+}$  ( $p > 0.05$ ) (Fig. 7C). However,  $[Ca^{2+}]_i$ , which normally plateaued following single spikes evoked by  $100 \text{ nM}$  AVP, tended to return slowly to the prestimulated level but was still above basal level  $1 \text{ min}$  after termination of the AVP application. Thus, step, oscillations, and spike patterns evoked by AVP did not depend on  $Ca^{2+}$  entry, but rather depended on  $Ca^{2+}$  release from intracellular  $Ca^{2+}$ -sequestering stores.

Experiments in which  $[Ca^{2+}]_i$  measurements were combined with patch-clamp recordings under current-clamp conditions revealed that  $[Ca^{2+}]_i$  oscillations and single spikes were closely associated with membrane hyperpolarization (Fig. 8). A monophasic hyperpolarizing response was observed in cells exhibiting a  $[Ca^{2+}]_i$  step upon AVP stimulation ( $n = 5$ , not shown). Furthermore, it should be noted that in more than  $50\%$  of cells (7 out of 13 cells recorded) the hyperpolarization was followed by a sustained depolarization that paralleled the plateau phase.

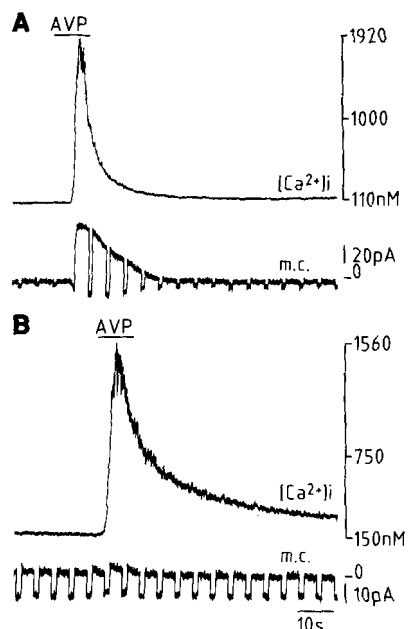
To investigate whether these  $Ca^{2+}$ -mobilizing responses to AVP could activate  $K^+$  channels to produce a hyperpolarization, experiments were conducted to monitor AVP-induced currents. When the membrane potential was held at  $-40 \text{ mV}$  under voltage-clamp conditions,  $Ca^{2+}$ -mobilizing responses to  $100 \text{ nM}$  AVP were closely correlated with an outwardly directed current (Fig. 9A). Periodic application of hyperpolarizing steps ( $-30 \text{ mV}$ ,  $2 \text{ s}$ ,  $0.2 \text{ Hz}$ ) revealed that an increase in membrane conductance paralleled the development of the outward current. From the relative changes in both outward



**FIG. 7. Effects of extracellular  $Ca^{2+}$  removal on  $[Ca^{2+}]_i$  step, oscillations, and spike/plateau patterns induced by AVP.** The figure shows  $[Ca^{2+}]_i$  recordings made in separate cells.  $Ca^{2+}$ -free medium (with 2 mM EGTA) was applied to cells just prior to AVP challenge as indicated by the horizontal bar. *A*,  $[Ca^{2+}]_i$  step produced in response to 1 nM AVP. *B*,  $[Ca^{2+}]_i$  oscillations produced in response to 10 nM AVP. *C*,  $[Ca^{2+}]_i$  spike produced in response to 100 nM AVP.



**FIG. 8. Membrane potential responses in AVP-stimulated cells presenting  $Ca^{2+}$ -mobilizing responses.** Combined  $[Ca^{2+}]_i$  and membrane potential recordings were obtained as in Fig. 6 in different corticotrophs. The top trace in each panel represents  $[Ca^{2+}]_i$ , and the bottom trace represents membrane potential. *A*, 10 nM AVP produced  $[Ca^{2+}]_i$  oscillations associated with an oscillating hyperpolarization. *B*, 100 nM AVP produced a  $[Ca^{2+}]_i$  spike associated with a transient hyperpolarization followed by a sustained depolarization.



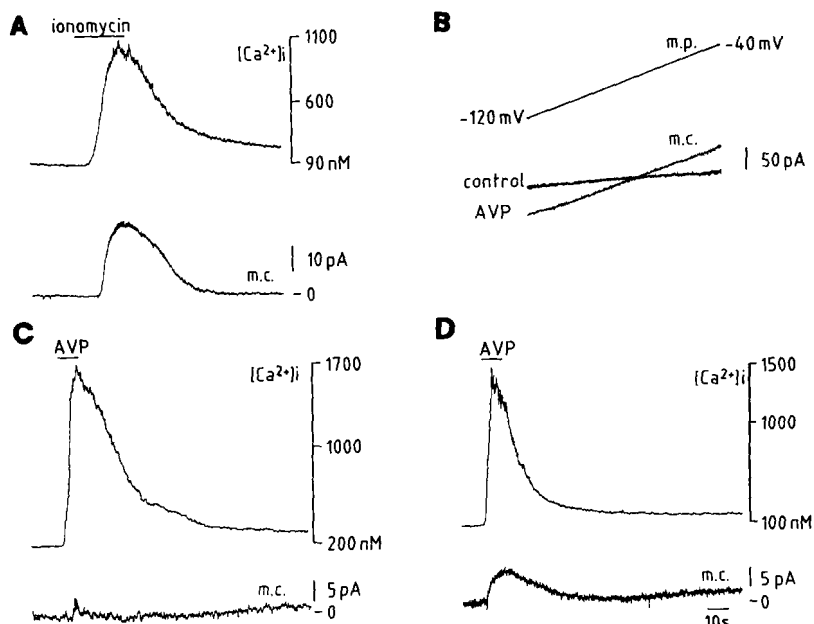
**FIG. 9. AVP-induced rise in  $[Ca^{2+}]_i$  and activation of outward  $K^+$  current monitored simultaneously in individual cells showing  $Ca^{2+}$ -mobilizing responses.** Combined recordings of  $[Ca^{2+}]_i$  and membrane current were obtained as in Fig. 6 in separate corticotrophs. The top trace in each panel represents  $[Ca^{2+}]_i$ , and the bottom trace represents membrane current measured under voltage clamp conditions with a holding potential of  $-40$  mV. Small, periodic excursions in holding potential ( $-30$  mV, 2 s, 0.2 Hz) served to monitor membrane conductance. *A*, 100 nM AVP produced a  $[Ca^{2+}]_i$  spike associated with an outward current and an increase in membrane conductance, as indicated by the increase in the current deflections evoked by the periodic potential steps. *B*, the 100 nM AVP-induced outward current and increase in membrane conductance were abolished when  $K^+$  channels were blocked by replacing  $K^+$  by  $Cs^+$  in the electrode solution.

current and membrane conductance during the  $Ca^{2+}$ -mobilizing response, the estimated reversal potential of the current (43) was  $-64.5$  mV, *i.e.* close to the  $K^+$  equilibrium potential. An additional indication that the outward current during the AVP-stimulated  $Ca^{2+}$  mobilization could have been carried by  $K^+$  is presented in Fig. 9*B*, in which  $K^+$  in the electrode solution was replaced by  $Cs^+$ , a  $K^+$  channel blocker. Under these conditions, the  $Ca^{2+}$  response to AVP was no longer associated with a change in either membrane current or conductance (Fig. 9*B*). Thus, these results are consistent with the notion that the  $Ca^{2+}$ -mobilizing response to AVP results in the activation of  $K^+$  channels that leads to a transient hyperpolarization.

Let us consider the AVP-induced  $K^+$  current response and assume that it is entirely due to activation of  $Ca^{2+}$ -dependent  $K^+$  channels. First, in corticotrophs preloaded with the calcium-chelating agent BAPTA, neither  $[Ca^{2+}]_i$  rise nor outward current responses to the peptide were observed ( $n = 7$ , not shown). Second, application of the calcium ionophore ionomycin ( $5 \mu M$ ) caused an outward current associated with a  $[Ca^{2+}]_i$  rise (Fig. 10*A*) ( $n = 5$ ). As upon AVP stimulation, no current was evoked in response to ionomycin when corticotrophs were preloaded with  $Cs^+$  ions, which were passively introduced into cells through nystatin-formed pores ( $n = 3$ ), suggesting that ionomycin-induced outward current, as AVP-evoked current response was due to the gating of  $Ca^{2+}$ -activated  $K^+$  channels. The voltage dependence of the conductance underlying the  $K^+$  current elicited by 100 nM AVP was obtained from experiments such as those shown in Fig. 10*B*, assuming that the speed of the voltage ramp (26 mV/s) is

### FIG. 10. Characterization of the AVP-induced $K^+$ current in cells exhibiting $Ca^{2+}$ -mobilizing responses.

Combined recordings of  $[Ca^{2+}]_i$  and membrane current were obtained as in Fig. 9 in separate corticotrophs. Cells shown in A and B mobilized intracellular  $Ca^{2+}$  in response to AVP prior to patch-clamping. A, application of the  $Ca^{2+}$  ionophore ionomycin ( $5 \mu M$ ) mimicked the AVP action in evoking a rise in  $[Ca^{2+}]_i$ , associated with the establishment of an outward current. B, voltage ramps were applied prior to response (Control), during the peak of the 100 nM AVP response (AVP), and after recovery. Note the relative linearity of the current-voltage relationship during AVP action. C, incubation of cells with  $1 \mu M$  apamin blocked the current, but not  $[Ca^{2+}]_i$  responses caused by 100 nM AVP. D, by contrast, 4 mM TEA failed to suppress the AVP-induced  $K^+$  current response.



sufficiently slow to allow time-dependent rectifying currents to develop ( $n = 3$ ). As may be anticipated from a close inspection of the curves in Fig. 10B, the conductance underlying the current evoked by AVP showed a weak voltage-dependency. Similar results were obtained in ionomycin-stimulated cells (2 out of 2 cells, not shown). We further investigated the nature of those  $Ca^{2+}$ -activated  $K^+$  channels using specific  $K^+$  channel blockers. As previously reported in other pituitary cells (44, 45),  $[Ca^{2+}]_i$  mobilizing responses mainly caused the activation of apamin-sensitive ("SK"-type)  $Ca^{2+}$ -activated  $K^+$  channels. When cells were incubated in the presence of  $1 \mu M$  apamin, 100 nM AVP could cause a typical spike but failed to evoke any outward current ( $n = 5$ ) (Fig. 10C). Similarly, incubation of cells with  $250 \mu M$  *d*-tubocurarine, another blocker of SK channels (46), prevented the stimulation of outward current but not  $[Ca^{2+}]_i$  spike to AVP ( $n = 10$ ) (not shown). By contrast, when cells were incubated with a low concentration of TEA (4 mM), which has been shown to be effective at blocking apamin-resistant  $Ca^{2+}$ -dependent  $K^+$  channels (44, 46), 100 nM AVP caused both  $[Ca^{2+}]_i$  spike and current responses (Fig. 10D) ( $n = 14$ ). Taken together, it seems likely that the  $[Ca^{2+}]_i$  mobilizing responses to AVP cause the activation of apamin-sensitive (SK)  $Ca^{2+}$ -activated  $K^+$  channels.

Note that the presence of  $Ca^{2+}$ -dependent  $K^+$  channels that can be activated at the resting potential is not unique to corticotrophs displaying  $[Ca^{2+}]_i$  mobilizing AVP responses. Indeed, in cells responding to AVP with a series of  $[Ca^{2+}]_i$  transients (Fig. 11A), a subsequent application of ionomycin under combined  $[Ca^{2+}]_i$  and patch-clamp experiments caused an outward potassium current that paralleled the  $[Ca^{2+}]_i$  rise (Fig. 11B) (6 out of 6 cells). Furthermore, the current, but not  $[Ca^{2+}]_i$  response was blocked when *d*-tubocurarine ( $250 \mu M$ ) was added to the ionomycin-containing solution while the  $Ca^{2+}$  response persisted (2 out of 2 cells; not shown).

**$Ca^{2+}$  Entry Is Involved in the Plateau Phase of the Spike Pattern.**—In the absence of extracellular  $Ca^{2+}$ , the  $[Ca^{2+}]_i$  tended to return slowly to basal level during the plateau phase of the AVP-induced response, suggesting that  $Ca^{2+}$  release from intracellular stores was only in part responsible for the plateau. To determine if  $Ca^{2+}$  entry was also involved in the plateau,  $Ca^{2+}$ -free medium was applied during this phase

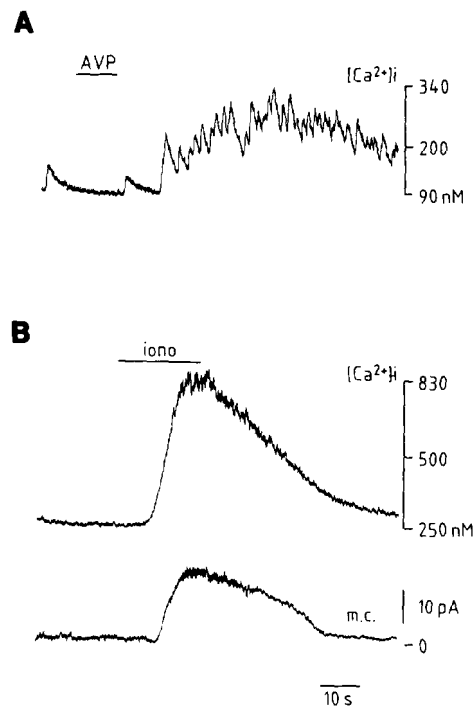
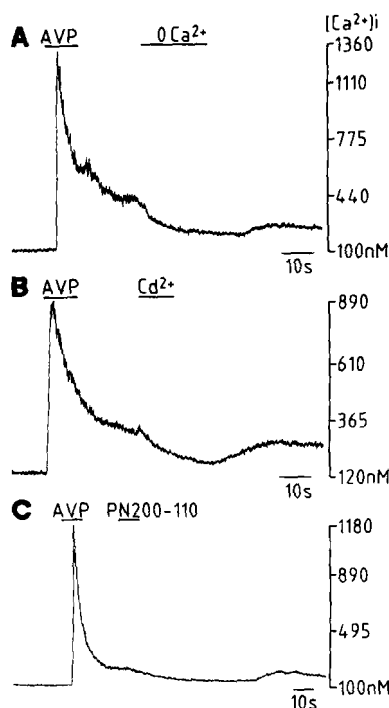
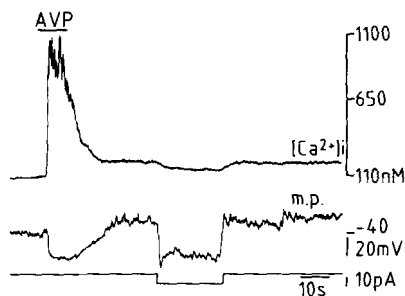


FIG. 11. Effects of ionomycin in a cell showing a  $[Ca^{2+}]_i$  transient pattern in response to AVP. A, during a  $[Ca^{2+}]_i$  recording in a single corticotroph, 100 nM AVP caused a typical series of  $[Ca^{2+}]_i$  transients. B, in the same cell that was subsequently voltage-clamped at a holding potential of  $-40$  mV,  $5 \mu M$  ionomycin caused an outward current that closely paralleled the evoked  $[Ca^{2+}]_i$  rise.

within 1 min after termination of the AVP application. Fig. 12A shows that the  $[Ca^{2+}]_i$  plateau was lowered, but not suppressed (mean level:  $352 \pm 102$  nM ( $n = 30$ ) versus  $180 \pm 49$  nM ( $n = 7$ ) for control versus  $Ca^{2+}$ -deprived cells;  $p < 0.05$ ) during the application of  $Ca^{2+}$ -free solution, and returned quickly to the plateau level when extracellular  $Ca^{2+}$  was restored. Similar results were observed with  $200 \mu M$   $Cd^{2+}$  ( $200 \pm 63$  nM for  $Cd^{2+}$ -treated cells ( $n = 7$ );  $p < 0.05$ ) (Fig. 12B). Furthermore, 100 nM PN 200-110 was effective at reducing the plateau phase ( $198 \pm 20$  nM ( $n = 10$ ) for PN 200-110-treated cells;  $p < 0.05$ ) (Fig. 12C). Since the plateau phase of



**FIG. 12. Effects of extracellular  $Ca^{2+}$  removal and  $Ca^{2+}$  channel blockers on the plateau phase of spike/plateau pattern.**  $[Ca^{2+}]_i$  recordings were carried out on separate cells in which 100 nM AVP evoked a  $[Ca^{2+}]_i$  spike/plateau response. *A*, the removal of extracellular  $Ca^{2+}$  significantly reduced the plateau. *B*, blocking  $Ca^{2+}$  entry with 200  $\mu M$  extracellular  $Cd^{2+}$  transiently and significantly lowered the plateau. *C*, blocking  $Ca^{2+}$  entry through L-type  $Ca^{2+}$  channels with 100 nM PN 200-110 significantly lowered the plateau.



**FIG. 13. Effects of membrane polarization on the plateau phase of spike/plateau pattern.** During combined  $[Ca^{2+}]_i$  and patch-clamp recordings (as in Fig. 6) in a corticotroph stimulated with 100 nM AVP, the membrane potential (*middle trace*) was polarized by adjusting current (*bottom trace*) during the evoked sustained depolarization. Concomitantly, there was a marked reduction of the  $[Ca^{2+}]_i$  plateau (*upper trace*).

the spike response was associated with a sustained membrane depolarization, we studied whether a membrane repolarization could curtail the  $[Ca^{2+}]_i$  plateau. When the membrane potential was polarized from  $-30$  to  $-65$  mV by injecting current 1 min after the cessation of peptide application, the  $[Ca^{2+}]_i$  plateau decreased slowly and monotonically (Fig. 13).  $[Ca^{2+}]_i$  did not return to prestimulated levels during the hyperpolarizing pulse. Upon termination of the current injection, membrane potential came back to its depolarized level and  $[Ca^{2+}]_i$  returned to its plateau level. Therefore,  $Ca^{2+}$  influx appears to be stimulated during the sustained depolarization, thus participates in the maintenance of the  $[Ca^{2+}]_i$  plateau.

#### DISCUSSION

The data presented here show that AVP can generate different cell-specific  $[Ca^{2+}]_i$  signals, which ultimately raise

$[Ca^{2+}]_i$  in normal rat corticotrophs. Cell to cell variation in agonist-triggered  $[Ca^{2+}]_i$  signals have been reported for many cell types (10, 47). In corticotrophs, not only does AVP produce different  $[Ca^{2+}]_i$  patterns, but the source of  $Ca^{2+}$  for each pattern and the variations in membrane potential associated with each pattern are different.

Experiments in which  $[Ca^{2+}]_i$  measurements were combined with perforated patch-clamp recordings demonstrated that the AVP-stimulated action potentials cause  $[Ca^{2+}]_i$  transients. The concerted opening of voltage-gated  $Ca^{2+}$  channels during a single action potential allows sufficient  $Ca^{2+}$  to enter to transiently increase  $[Ca^{2+}]_i$ . Similar paradigms of  $[Ca^{2+}]_i$  regulation via electrical activity have been found in other pituitary cells (19), including tumoral corticotrophs (18, 48). We show that AVP-stimulated  $[Ca^{2+}]_i$  transients were markedly reduced and suppressed by the dihydropyridine antagonist PN 200-110 and  $Cd^{2+}$ , respectively, supporting the notion that both dihydropyridine-sensitive and dihydropyridine-insensitive  $Ca^{2+}$  channels are activated during AVP-evoked action potentials. In corticotrophs, dihydropyridine-sensitive  $Ca^{2+}$  channels correspond to voltage-gated  $Ca^{2+}$  channels of the L-type that allow a rapidly activating, slowly inactivating  $Ca^{2+}$  entry with a high voltage threshold (18, 49). Furthermore, the voltage-gated T-type  $Ca^{2+}$  channels, which, in corticotrophs, are responsible for rapidly activating, rapidly inactivating  $Ca^{2+}$  currents with a low threshold (18, 49) could be potential candidates for the dihydropyridine-insensitive,  $Cd^{2+}$ -sensitive  $Ca^{2+}$  entry involved in AVP-stimulated  $[Ca^{2+}]_i$  transient patterns. In cells where  $[Ca^{2+}]_i$  transients are blocked by the  $Na^+$  channel blocker TTX,  $Na^+$  channels are probably responsible for the establishment of action potentials. In the remainder, the triggering of electrical activity would be due to the opening of low threshold voltage-gated T-type  $Ca^{2+}$  channels (18, 49).

The results of this study indicate that  $[Ca^{2+}]_i$  steps, oscillations, and spikes evoked by AVP, in a subset of corticotrophs, depend on  $Ca^{2+}$  release from intracellular  $Ca^{2+}$ -sequestering stores. The spike pattern probably corresponds to the previously reported  $[Ca^{2+}]_i$  spike produced by high AVP concentrations (100 nM to 1  $\mu M$ ) in single corticotrophs (38, 39). Interestingly, the transition from oscillations to spike that was observed upon raising AVP concentration in some cells resembles the concentration-dependent shift described in gonadotropin-releasing hormone (GnRH)-stimulated gonadotrophs (7, 9, 50, 51). Although AVP can elicit fewer (two or three) oscillations in corticotrophs than GnRH in gonadotrophs, it seems likely that some corticotrophs can encode AVP concentration by shifting between  $[Ca^{2+}]_i$  oscillations and spike (8). It is now clear that gonadotrophs are not the sole pituitary cell type that shows  $Ca^{2+}$ -activated  $K^+$  channel-dependent hyperpolarizing oscillations (45). In corticotrophs, AVP-dependent membrane potential oscillations and monophasic hyperpolarization are due to an increase in  $K^+$  conductance that closely parallels changes in  $[Ca^{2+}]_i$ . The change in  $K^+$  conductance depends on the opening of  $Ca^{2+}$ -activated  $K^+$  channels, which have a weak voltage dependence (44, 45, 52) and are blocked by the bee venom toxin apamin (44–46, 53, 54). Therefore, as in gonadotrophs and clonal GH3 cells, the initial change in  $[Ca^{2+}]_i$  that is responsible for the activation of apamin-sensitive  $Ca^{2+}$ -activated  $K^+$  channels (SK-type) is attributable to the  $Ca^{2+}$ -mobilizing effect of the secretagogue (44, 45, 52–54).

Data from combined  $[Ca^{2+}]_i$  and patch-clamp experiments strongly support the suggestion that in corticotrophs showing a spike response, AVP evoked a late and prolonged  $Ca^{2+}$  entry during a sustained depolarization. It seems likely that this  $Ca^{2+}$  entry participates in the  $[Ca^{2+}]_i$  plateau that follows the

$[Ca^{2+}]_i$  spike. Although AVP, in these cells, did not initiate action potentials and their associated  $Ca^{2+}$  influx, it did produce a small and maintained depolarization within  $-55$  to  $-35$  mV, during which a sustained dihydropyridine-sensitive  $Ca^{2+}$  influx occurred. This implies that the dihydropyridine-sensitive  $Ca^{2+}$  channels carrying this  $Ca^{2+}$  influx must differ from the high threshold ( $-25$  mV) dihydropyridine-sensitive channels, which are transiently opened during action potentials (49, 55). Recently, a small, low threshold ( $-60$  mV), steady-state  $Ca^{2+}$  current sensitive to dihydropyridines has been observed in pituitary GH3 cells (56). Thus, it is possible that AVP would favor the opening of a subtype (57) or another conductive mode (58, 59) of dihydropyridine-sensitive (L-type)  $Ca^{2+}$  channels to produce a sustained  $Ca^{2+}$  influx. This  $Ca^{2+}$  entry and its associated rise in  $[Ca^{2+}]_i$  is not associated with the depletion of internal  $Ca^{2+}$ -sequestering stores. Indeed, a " $Ca^{2+}$  release-activated  $Ca^{2+}$  current" ( $I_{crac}$ ) (60) appears not to be activated upon AVP stimulation. If such a non-voltage-gated  $Ca^{2+}$  current was involved in the post-spike plateau, it is expected that hyperpolarization, which increases the driving force for  $Ca^{2+}$  ions, should lead to an increase in  $Ca^{2+}$  current and  $[Ca^{2+}]_i$ . This is clearly not the case in corticotrophs (see Fig. 13). Note, however, that the lack of evidence for the participation of an  $I_{crac}$ -like current in the  $[Ca^{2+}]_i$  plateau does not preclude the possibility that the maintenance of internal AVP-sensitive stores depends on  $Ca^{2+}$  entry from the external milieu (61, 62).

The plateau phase is not solely dependent on  $Ca^{2+}$  entry. Over the observation period of several minutes, in the absence of extracellular  $Ca^{2+}$ ,  $[Ca^{2+}]_i$  does not return to basal after the spike event though a monotonic decrease in  $[Ca^{2+}]_i$  is observed. This indicates that the rate of  $Ca^{2+}$  sequestration is low. The prolonged period of slightly elevated  $[Ca^{2+}]_i$  resembles the slow component of  $Ca^{2+}$ -mobilizing responses found in other secretory cells (63, 64).

The data clearly show that AVP can produce distinct  $[Ca^{2+}]_i$  signals that are linked to at least two electrical events. If one recalls that corticotrophs possess only one type of AVP receptor, the question of how it can mediate multiple  $[Ca^{2+}]_i$  signals arises. Distinct G-proteins could be responsible for coupling a single receptor to several second message pathways (65–67). Gollash *et al.* (68) have recently shown, in GH3 cells, that thyrotropin-releasing hormone causes two modes of  $[Ca^{2+}]_i$  increase and related electrical events through distinct G-proteins. It remains to be established whether the transduction mechanisms (Ins-1,4,5- $P_3$ , protein kinase C, arachidonic acid) known to be activated in AVP-stimulated cells (31–36) are involved in the  $[Ca^{2+}]_i$  signals linked to AVP stimulation.

The physiological relevance of AVP  $[Ca^{2+}]_i$  responses may be viewed in terms of  $[Ca^{2+}]_i$  signal and ACTH secretion patterns. A recent single cell secretion study using reverse hemolytic plaque assay has shown that a single corticotroph could exhibit nongraded (all-or-none) ACTH release upon AVP stimulation and exhibit graded secretion in response to CRF (69). The authors have proposed that Ins-1,4,5- $P_3$ -sensitive  $Ca^{2+}$  release from internal stores may act as a trigger for AVP-stimulated all-or-none secretion. This scheme would be applicable only to corticotrophs that raise their  $[Ca^{2+}]_i$  in response to AVP by releasing sequestered  $Ca^{2+}$  and not to those that increase  $Ca^{2+}$  influx by increasing electrical activity. Since both AVP and CRF can generate  $[Ca^{2+}]_i$  transients, which are virtually identical with respect to the underlying electrical activity and source, the difference in secretion patterns may be related to the different second messengers involved. Alternatively,  $Ca^{2+}$ -dependent proteins, notably

$Ca^{2+}$ /calmodulin-dependent protein kinases, could differentially decode the amplitude and frequency components of the AVP-stimulated  $[Ca^{2+}]_i$  signal (70–72). Single cell secretory measurements using patch-clamp monitoring of membrane capacitance (73) will allow us to answer the question of how a rise in  $[Ca^{2+}]_i$  due to either voltage-gated  $Ca^{2+}$  entry or release from internal  $Ca^{2+}$  sequestering stores may trigger the exocytotic process in corticotrophs (2, 74, 75). With respect to the investigation of the role of AVP-induced  $[Ca^{2+}]_i$  responses in the activation of gene expression and cell proliferation, polymerase chain reaction and *in situ* hybridization can be applied in individual corticotrophs following patch-clamp and fluorescent  $[Ca^{2+}]_i$  measurements (76, 77).

In summary, we have presented evidence that single corticotrophs generate different  $[Ca^{2+}]_i$  signals in response to AVP. These  $[Ca^{2+}]_i$  responses that involve internal  $Ca^{2+}$  mobilization and/or  $Ca^{2+}$  entry are closely associated with distinct changes in ionic channel activities. This heterogeneous responsiveness to AVP with regard to the pattern and origin of  $[Ca^{2+}]_i$  responses contrasts with the unique CRF  $[Ca^{2+}]_i$  signal.

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