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# INTRACELLULAR CALCIUM AND VASOPRESSIN RELEASE OF RAT ISOLATED NEUROHYPOPHYSIAL NERVE ENDINGS

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SUMMARY

1. Monitoring of $[Ca^{2+}]_i$ and vasopressin secretion in isolated nerve endings from the rat neurohypophysis were studied to determine the relationship between the time course of vasopressin secretion and depolarization-induced changes in $[Ca^{2+}]_i$.

2. Membrane depolarization by increasing the extracellular $[K^+]$ led to concentration-dependent, parallel increases in the amount of vasopressin release and in peak increases in $[Ca^{2+}]_i$. Half-maximal activation of a change in $[Ca^{2+}]_i$ was attained at 40 mM extracellular $K^+$.

3. The $Ca^{2+}$ chelator dimethyl-BAPTA (1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N'N'*-tetraacetic acid), loaded into the nerve endings, reduced $K^+$ depolarization-evoked vasopressin release and efficiently antagonized $K^+$-induced changes in $[Ca^{2+}]_i$. Moreover, dimethyl-BAPTA dramatically reduced basal $[Ca^{2+}]_i$ without a reduction in basal secretion.

4. The duration of the vasopressin secretory response was similar regardless of applied 50 mM $K^+$ depolarizations longer than 30 s. The $t_{\frac{1}{2}}$ of the secretory response was 45 s. Application of repetitive $K^+$ depolarization pulses produced repetitive secretory responses of similar amplitude and duration.

5. The $K^+$-induced changes in $[Ca^{2+}]_i$ remained elevated throughout the duration of the depolarizing stimulus decreasing less than 30% over 3 min. The sustained increase in $[Ca^{2+}]_i$ resulted largely from continued enhanced $Ca^{2+}$ influx, demonstrated by susceptibility to the dihydropyridine, L-type calcium channel blocker, nicardipine.

6. Vasopressin secretion could be reinitiated following its decline to a step $K^+$ depolarization by a further step increase in $K^+$ or by removal and readdition of extracellular $[Ca^{2+}]$. Alterations in $[Ca^{2+}]_i$ paralleled periods of secretory activity.

7. Analysis of secretory responsiveness and change in $[Ca^{2+}]_i$ to $K^+$ depolarization in medium of altered extracellular $[Ca^{2+}]$ indicates that $[Ca^{2+}]_i$ of 20 $\mu$M is sufficient to trigger vasopressin release. $K^+$-induced alterations in $[Ca^{2+}]_i$ could be observed at $[Ca^{2+}]_o$ as low as 5 $\mu$M. Although smaller in amplitude to that observed at 2·2 mM $[Ca^{2+}]_o$ the duration of the $K^+$-induced secretory response increased at lower $[Ca^{2+}]_o$.

8. Transient vasopressin secretory responses were observed to sustained levels of $[Ca^{2+}]$ in digitonin and streptolysin-O-permeabilized nerve endings. Secretion could

be re-evoked, following its decline, by a step increase in $[Ca^{2+}]$ or by removal and readdition of $[Ca^{2+}]_o$.

9. These results show that the amount and duration of depolarization-induced vasopressin secretion from isolated nerve endings may be regulated not only by the absolute increase but also by periodic changes in $[Ca^{2+}]_i$.

## INTRODUCTION

The entry of calcium, in response to impulse-mediated membrane depolarization and activation of potential-sensitive $Ca^{2+}$ channels, is largely believed to be necessary and sufficient to initiate release of chemical messengers from nerve endings. The supporting evidence arises from extensive studies in a variety of neuronal and nerve ending preparations (Augustine, Charlton & Smith, 1987; Kelly, 1988). However, in spite of quantitative detail, predominated specifically by studies at the squid giant synapse (Llinás, Steinberg & Walton, 1976; Augustine, Charlton & Smith, 1985), various spatiotemporal considerations suggest that the relationship between inward calcium current and release may be a too simplistic description of the secretory process at nerve endings (Llinás, 1991). For example, nerve endings may possess locally restricted sites for exocytotic release with $Ca^{2+}$ channels clustered near release sites (Pumplin, Reese & Llinás, 1981). In addition, multiple $Ca^{2+}$ channel types in vertebrate neurons, differing in activation and inactivation properties, may co-exist in nerve endings and be differently related to the secretory process (Miller, 1987; Hirning *et al.* 1988).

While the increase in cytoplasmic calcium concentration triggers release, the time course of secretion of the chemical messenger(s) from nerve endings, as given by the calcium hypothesis, is considered a function of the rapid dissipation of the rise in intracellular calcium concentration ($[Ca^{2+}]_i$) near exocytotic release sites. However, studies which have utilized $Ca^{2+}$-sensitive indicators to monitor stimulus-induced changes in $[Ca^{2+}]_i$ in nerve endings have reported a decline in release manyfold faster than $[Ca^{2+}]_i$ (Llinás & Nicholson, 1975; Miledi & Parker, 1981). Transient secretory responses to sustained stimulation have also been reported for chromaffin cells (Douglas & Rubin, 1963; Baker & Rink, 1975) and sympathetic neurones (García, Kirpekar & Sánchez-García, 1976). The presence of phasic secretory responses in the face of continued stimulation has most often been attributed to a decline in $Ca^{2+}$ conductance resulting from inactivation of the voltage-dependent $Ca^{2+}$ channels (Nordmann, 1976; Birman & Meunier, 1985). The apparent discrepancy between the duration of the secretory response and that of the change in $[Ca^{2+}]_i$ has, therefore, been suggested to be the result of inadequate spatiotemporal resolution of $[Ca^{2+}]_i$ at release sites (Simon & Llinás, 1985; Zucker, Delaney, Mulkey & Tank, 1991; Augustine, Adler & Charlton, 1991). Thus, the sharp $[Ca^{2+}]_i$ gradients that are transiently produced at the release site during impulse invasion are under represented by a $Ca^{2+}$ signal averaged over the entire nerve ending. Alternatively, there may be intrinsic components of the release process which act to regulate the secretory time course such as exhaustion of an immediately available releasable portion of the secretory vesicles or granules, different $Ca^{2+}$ sensitivities of releasable pools, exhaustion of a primed substrate, feedback inhibition by a $Ca^{2+}$-sensitive

intermediate or desensitization of the release apparatus to $Ca^{2+}$ (for review see Knight & Baker, 1982).

In the present study we have re-examined the correlation between depolarization-induced changes in averaged $[Ca^{2+}]_i$ and vasopressin (AVP) secretion from a preparation of isolated neurohypophysial nerve endings. We have utilized methodological approaches which allow evoked changes in $[Ca^{2+}]_i$, measured in single isolated nerve endings, to be correlated to AVP secretion on a time resolved scale of seconds. We have observed, consistent with observations on other secretory systems (Baker & Rink, 1975; Llinás & Nicholson, 1975; Miledi & Parker, 1981; Cheek, Jackson, O'Sullivan, Moreton, Berridge & Burgoyne, 1989), that a decline in AVP secretion occurs despite a sustained increase in $[Ca^{2+}]_i$ in both intact and permeabilized neurohypophysial nerve endings. However, our results suggest that, in the neurohypophysis, the decline in AVP secretion cannot be explained solely on the basis of a decline in $Ca^{2+}$ conductance of the nerve ending coupled with inadequate spatiotemporal resolution of $Ca^{2+}$ dynamics, or depletion of an immediately releasable store of granules or different sensitivities of $Ca^{2+}$ receptors. We conclude by suggesting that, in addition to some of the components above, the rate and extent of secretion may be regulated not only by the absolute increase in $[Ca^{2+}]_i$ but also by periodic changes in $[Ca^{2+}]_i$. A preliminary report of portions of this work has appeared in abstract form (Stuenkel & Nordmann, 1991).

## METHODS

### *Preparation of isolated nerve endings*

Isolated nerve endings were prepared from male Sprague–Dawley or Wistar rats (225–280 g) as previously described (Cazalis, Dayanithi & Nordmann, 1987*a*). The animals were killed by decapitation preceded in some cases by $CO_2$ asphyxiation. Following removal of the pars intermedia the neural lobe was homogenized in a buffer at 37 °C containing (mM): sucrose, 270; EGTA, 2; Hepes, 10; buffered to pH 7·2 with Tris. In a number of experiments the EGTA concentration in the homogenization solution was 10 $\mu$M, as originally reported by Cazalis *et al.* (1987*a*). No differences were observed in either $[Ca^{2+}]_i$ or AVP release experiments between the two solutions. For release experiments the homogenate was then spun at $100 \times g$ for 1 min with the supernatant then spun at $2400 \times g$ for 4 min. The resulting pellet was then resuspended in normal physiological saline at 37 °C containing (mM): NaCl, 140; $KHCO_3$, 5; $MgCl_2$, 1; $CaCl_2$, 2·2; glucose, 10 and Hepes, 10, buffered to pH 7·2 with Tris; bovine serum albumin (BSA), 0·01 %. For $[Ca^{2+}]_i$ monitoring experiments the initial low speed centrifugation was omitted and the pellet was resuspended in a physiological saline at 37 °C containing (mM): NaCl, 140; KCl, 5; $CaCl_2$, 2·2; $MgCl_2$, 1; glucose, 10 and NaOH-Hepes, 10, buffered to pH 7·2; BSA 0·01 %. In both cases a highly purified suspension of isolated nerve terminals containing either vasopressin or oxytocin was produced (Cazalis *et al.* 1987*a*). While the vast majority of the isolated nerve endings are less than 2 $\mu$m in diameter there occur several hundreds of large diameter of which those in the 4–8 $\mu$m range were used for the $[Ca^{2+}]_i$ measurements.

The physiological saline (termed hereafter 40 mM $Na^+$ saline) used for perfusion in both the AVP release and $[Ca^{2+}]_i$ monitoring experiments contained (mM): *N*-methyl-D-glucamine Cl, 100; NaCl, 40; KCl, 5 (or $KHCO_3$, 5); $CaCl_2$, 2·2; $MgCl_2$, 1; glucose, 10 and Tris- or NaOH-Hepes, 10. In this manner solutions of elevated $[K^+]$ were prepared by equimolar substitution of the *N*-methyl-D-glucamine Cl without altering the $[Na^+]_o$. Different free $[Ca^{2+}]$ were obtained by use of appropriate $Ca^{2+}$ buffers such as EGTA, HEDTA (hydroxyethylenediaminetetraacetic acid), and NTA (nitrilotriacetic acid). The $Ca^{2+}$/buffer ratios were determined by taking into account the binding of $Ca^{2+}$ and $Mg^+$. Absolute stability constants determined for an ionic strength of 0·1 M (Martell & Smith, 1974) were converted into apparent stability constants, taking into account the $H^+$ activity

and temperature. Each solution was carefully monitored and adjusted, if necessary, to maintain osmolarity between 295 and 310 mosmol $l^{-1}$.

*Determination of AVP release by fast flow perfusion*

Following loading of the nerve endings onto filters (0·45 $\mu$m Acrodisc LCPVDF, Gelman) they were perfused (Minipulse Peristaltic Pump, Gilson, Middleton, WI, USA; 50 $\mu$l $min^{-1}$) for 40 min with normal physiological saline followed by 40 min of the 40 mM $Na^+$ saline. The flow was then increased over a 20 min period to *ca* 1·2 ml $min^{-1}$. The perfusate was collected (5 drops, i.e. 100 $\mu$l per tube) and the perfusate solutions changed *via* a manual valve (Rainin, Woburn, MA, USA) according to the experimental protocol. Generally, AVP content in every second fraction was determined by radioimmunoassay as described (Cazalis, Dayanithi & Nordmann, 1985). Alternative to utilizing a peristaltic pump a low pressure (< 14 kPa above ambient) pneumatic system (Omnivalve, Rainin Instrument, Woburn, MA, USA) was found to be suitable for these studies. Differences in the total amount of AVP released between experiments results from differences in the amount of each preparation loaded onto the filters and from heterogeneity of secretory responsiveness between preparations.

*Permeabilization of nerve endings*

After loading the nerve endings on a filter and perfusion with normal physiological saline first in the presence of 2·2 mM $Ca^{2+}$ (40 min) then in the absence of $Ca^{2+}$ (2 mM EGTA no added $Ca^{2+}$; 10 min) they were then perfused (30 min) with medium containing (mM) potassium glutamate, 140; $MgCl_2$, 2; EGTA, 2; glucose, 10; Tris-Pipes (1,4-piperazinediethanesulphonic acid), 10 adjusted to pH 6·8. The nerve endings were then permeabilized with either digitonin (2 $\mu$M) or streptolysin-O (100 i.u. $ml^{-1}$) added to the perfusing medium (50 $\mu$l $min^{-1}$) for a period of 5 min. Collection of the fast flow perfusate (1·2 ml $min^{-1}$; see above) was started 30–40 min following permeabilization.

*Monitoring of* $[Ca^{2+}]_i$

For loading of the $Ca^{2+}$-sensitive, fluorescent indicator fura-2 isolated nerve terminals were incubated for 20 min at 37 °C with 1·5 $\mu$M fura-2 AM. After fura-2 loading the nerve endings were centrifuged at 2400 *g* for 4 min to remove extracellular indicator and resuspended in normal physiological saline. An incubation period of approximately 25 min at 37 °C was then given to allow for cleavage of intracellularly accumulated fura-2 AM. Monitoring of $[Ca^{2+}]_i$ in individual nerve endings was performed by dual wavelength microspectrofluorometry similar to that described previously (Stuenkel, 1990; Lindau, Stuenkel & Nordmann, 1992). Briefly, an aliquot of the nerve endings was loaded into a chamber (65 $\mu$l volume) and allowed to adhere to a cover glass forming the base of the chamber before beginning rapid exchange of the superfusing physiological saline (1·5 ml $min^{-1}$). With the exception of the digital imaging experiments monitoring of fura-2 fluorescence was as follows. Selection of a single nerve ending was performed and masked from surrounding regions by a pinhole diaphragm stopped down to a diameter of 5 or 10 $\mu$m. Alternating excitation wavelengths of $340 \pm 10$ and $380 \pm 10$ nm and monitoring of emitted light at $510 \pm 10$ nm, or in some cases at $500 \pm 20$ nm, was performed by a photomultiplier-based SPEX Industries (Edison, NJ, USA) AR-CM system. As the emitted signal at each wavelength represents the averaged intensity (0·3 to 0·5 s per sample) collected over the entire nerve ending, the calculated $[Ca^{2+}]_i$ value represents a spatially averaged value. Digital imaging of fura-2 fluorescence was performed using an Attofluor (Zeiss, Thornwood, NY, USA) imaging system. The fluorescence ratio (340/380 nm) in both cases was converted to $[Ca^{2+}]_i$ using the equation of Grynkiewicz, Poenie & Tsien (1985). Values of $R_{min}$, $R_{max}$ and $F_o/F_s$ were determined using an external standard calibration technique where $R_{min}$ and $R_{max}$ are the values of $R$ at limiting and saturating $Ca^{2+}$ concentrations, respectively, and $F_o/F_s$ represents the ratio of emitted fluorescence intensity at 380 nm excitation at the limiting ($F_o$) and saturating ($F_s$) $Ca^{2+}$ levels. A $K_d$ (dissociation constant) value for Fura-2 of 224 nM was taken from the literature (Grynkiewicz *et al.* 1985). Autofluorescence, determined on unloaded nerve endings, was found to be less than 10% of the emitted fluorescent signal and was not subtracted. It should be noted that there are a number of potential errors associated with determination of the $[Ca^{2+}]_i$ values including defining the appropriate $K_d$ for cellular fura-2, compartmentilization of the dye, changes in autofluorescence or generation of fluorescent, $Ca^{2+}$-sensitive radicals (Williams & Fay, 1990). Loading the nerve endings with fura-2 AM at 20–25 °C to reduce incorporation into endocytotic vesicles resulted in no apparent differences in the $[Ca^{2+}]_i$

measurements. No punctate fluorescence nor elevated $[K^+]$ changes in autofluorescence have been observed. However, based on incomplete information regarding the additional considerations the $[Ca^{2+}]_i$ values reported here should be regarded as a best estimate of the actual averaged $[Ca^{2+}]_i$.

*Electrophysiological recording*

Conventional whole-cell patch clamp methods were used (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The pipette and bath solutions were constructed to isolate as much as possible evoked $Ca^{2+}$ currents from other ionic conductances. The bath solution was composed of (mM): tetraethylammonium, 140; $CaCl_2$, 10; Tris-Hepes, 10; glucose, 19; with pH adjusted to 7·2. The pipette solution contained (mM): *N*-methyl-D-glucamine, 140; Mg-ATP, 2; Tris-Hepes, 40, adjusted to pH 7·2; to which 100 $\mu$M EGTA and 150 $\mu$M fura-2 were added. Whole-cell recordings were obtained from nerve endings where seal resistances > 10 GΩ were achieved. Recorded currents were filtered at 10 kHz, −3 dB corner frequency, and digitized at 6·7 kHz simultaneous with recording of fura-2 fluorescence changes (5 Hz) as described above.

*Data analysis*

The data presented in this paper are based on intracellular calcium measurements from individual nerve endings while the secretory data rely on radioimmunoassay measurement of vasopressin release from populations of isolated nerve endings. The measurements of $[Ca^{2+}]_i$ and AVP release were performed on independent preparations although care was taken to match chamber turnover times to allow close correlation of the results. The $t_{\frac{1}{2}}$ of the secretory response was calculated as the duration taken from onset of the secretory response to one-half the peak amplitude on the falling phase of the secretory response. Results are presented as means ± S.E.M. with the number of observations given in parenthesis.

## RESULTS

### *Dependence of AVP secretion on* $[Ca^{2+}]_i$

Figure 1 illustrates the effect of altering the membrane potential with external potassium on $[Ca^{2+}]_i$. The induced change in $[Ca^{2+}]_i$ increases as a steep function of $[K^+]_o$ exhibiting a half-maximal increase at 40 mM. The data represent mean $[Ca^{2+}]_i$ values at the peak response obtained from observations of single nerve endings ($46 \geqslant n \geqslant 6$). As the $[Ca^{2+}]$ determinations are based on direct photon counting of emitted fluorescence of fura-2 over an entire nerve ending ($\leqslant 8$ $\mu$m diameter) the values represent a spatially averaged $[Ca^{2+}]_i$. The averaged $[Ca^{2+}]_i$ values, therefore, underestimate localized domains of elevated $[Ca^{2+}]_i$ which presumably occur near the sites of $Ca^{2+}$ entry at the plasma membrane. Attempts to spatially resolve fura-2 fluorescence of nerve endings between 4 and 8 $\mu$m in diameter, using digital video imaging, also showed rapid equilibration of $[Ca^{2+}]_i$ to a depolarizing stimulus. Spatial gradients of $[Ca^{2+}]_i$ were not observed during the depolarizing stimulus, in spite of continued $Ca^{2+}$ influx (see below). The use of depolarizing stimuli lasting tens of seconds, which is 100 fold greater than theoretical estimates of $[Ca^{2+}]_i$ equilibration for a 10 $\mu$m diameter cell (Delaney, Zucker & Tank, 1989), may account for a $[Ca^{2+}]_i$ distribution that is nearly uniform throughout the nerve ending.

The dependence of $K^+$-evoked secretion of AVP on an evoked increase in $[Ca^{2+}]_i$ is illustrated in Fig. 2. In these studies nerve endings were preincubated for a period of 30 min with the $Ca^{2+}$ chelator dimethyl-BAPTA AM (50 $\mu$M) which competes with intrinsic $Ca^{2+}$ receptors for $Ca^{2+}$ brought in by the depolarizing stimulus (Augustine *et al.* 1991). The BAPTA-loaded nerve endings showed a greatly diminished secretory response (Fig. 2*A*) and complete elimination of an increase in $[Ca^{2+}]_i$ (Fig. 2*B*) when challenged with a pulse of 50 mM $K^+$ as compared to control endings from the same

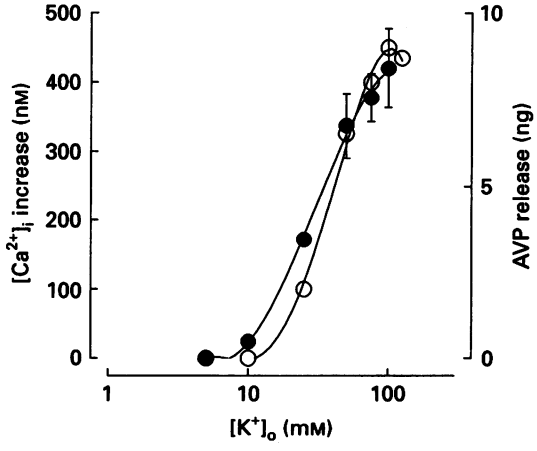


Fig. 1. Comparison of the amplitude change in $[Ca^{2+}]_i$ with the peak release of AVP to alteration of external $[K^+]$ in isolated nerve endings. AVP release data (○; means only) taken from Cazalis *et al.* (1987*a*). The $[Ca^{2+}]_i$ (●; means ± S.E.M.) represent the peak values attained in response to a greater than 30 s $K^+$ depolarization. AVP release was performed under $Na^+$-free (choline-substituted) conditions and represents the amount of AVP released during a 10 min period of elevated $K^+$ depolarization. In preparations receiving sequential elevated $[K^+]$ stimulations the data were considered valid only if the first and last stimulation at the same $[K^+]$ gave equivalent increases in $[Ca^{2+}]_i$ or AVP release.

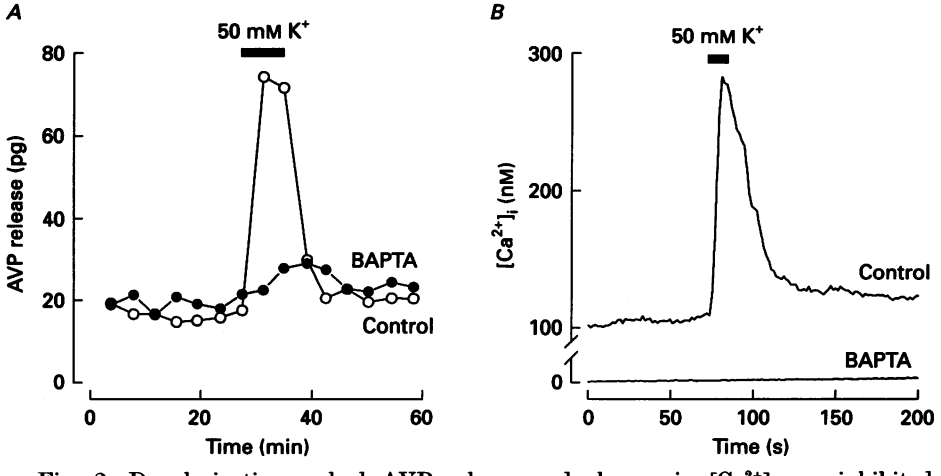


Fig. 2. Depolarization-evoked AVP release and change in $[Ca^{2+}]_i$ are inhibited in dimethyl-BAPTA-loaded nerve endings. Comparison of $K^+$ depolarization on AVP secretory response (*A*) and change in $[Ca^{2+}]_i$ (*B*). AVP release (*A*) from control (○) and dimethyl-BAPTA (●) -loaded endings monitored under reduced flow rate (50 $\mu$l $min^{-1}$). Control and BAPTA-loaded results were obtained on aliquots of the same preparation of nerve endings for *A* and for *B*.

preparations. Mean evoked AVP release to 50 mM $K^+$ depolarization under control and BAPTA-loaded conditions was 159 ± 9 pg ($n = 3$) and 49 ± 9 pg ($n = 4$) respectively. The large reduction in evoked AVP release in BAPTA-loaded endings

suggests that BAPTA can efficiently compete with the exocytotic $Ca^{2+}$ receptor for free $Ca^{2+}$ near the release site. Dimethyl-BAPTA ($K_d$ = 40 nM) also reduced averaged $[Ca^{2+}]_i$ levels ($n$ = 10) in the nerve terminals to below that accurately reported by fura-2 ($K_d$ = 224 nM). A correlated reduction in basal AVP release from BAPTA-

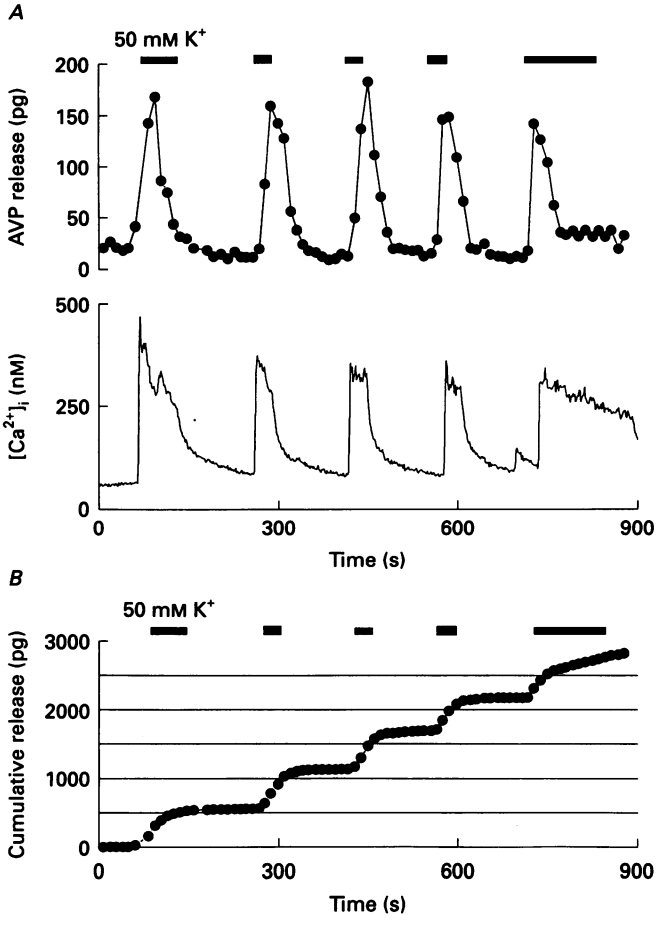


Fig. 3. Effect of repetitive $K^+$ depolarization on AVP release and $[Ca^{2+}]_i$. *A*, comparison of AVP release (upper) and $[Ca^{2+}]_i$ (lower) responses to repetitive pulses of 50 mM $K^+$. Periods of $K^+$ depolarization indicated by filled bar with durations of 60 s for the first periods, 30 s for the second to fourth periods and 150 s for the last period. *B*, cumulative AVP release corresponding to data presented in *A*.

loaded nerve endings was not observed ($17{\cdot}7 \pm 2{\cdot}3$ pg $min^{-1}$, control *versus* $19{\cdot}5 \pm 1{\cdot}9$ pg $min^{-1}$, BAPTA-loaded; $3 \leqslant n \leqslant 4$) suggesting that basal AVP secretion, under these conditions, may be dependent upon factors in addition to $[Ca^{2+}]_i$ (Toescu & Nordmann, 1991). Treatment with dimethyl sulphoxide, used as carrier for BAPTA AM, had no effect on the secretory response ($n$ = 3) or induced changes in $[Ca^{2+}]_i$ ($n$ = 3).

*Temporal correlation between AVP release and* $[Ca^{2+}]_i$

Figure 3 shows a representative example of the effect of repetitive 50 mM $K^+$ stimulation on AVP release and $[Ca^{2+}]_i$. Each stimulation induced a quantity of secreted AVP and a change in $[Ca^{2+}]_i$ of approximately equivalent amplitude

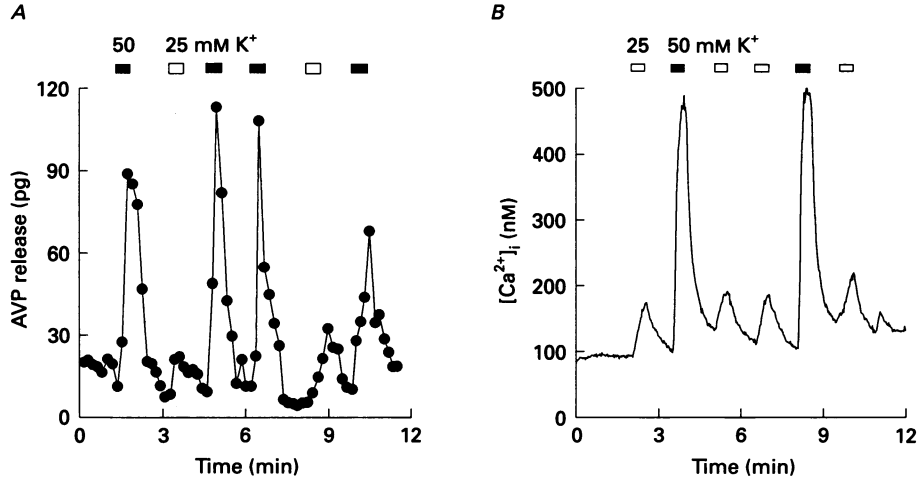


Fig. 4. Comparison of $K^+$ depolarizations of differing strengths on AVP release and $[Ca^{2+}]_i$. The effect of 25 mM $[K^+]_o$ alternating with 50 mM $[K^+]_o$ on the amplitude and time course of AVP release (*A*) and $[Ca^{2+}]_i$ (*B*).

(Fig. 3*A*). This suggests that there is no desensitization of either the induced rise in $[Ca^{2+}]_i$ or of the secretory process and also that there is availability of releasable vasopressin. Application of alternating periods of 25 and 50 mM $K^+$ stimulation led to repetitive secretory responses and changes in $[Ca^{2+}]_i$ that were directly related to the strength of the $K^+$ depolarization (Fig. 4).

A dichotomy between the secretory response and $[Ca^{2+}]_i$, however, was observed on application of $K^+$-depolarizing stimuli for longer than 30 s as is shown in the first (60 s) and last pulse periods (150 s) in Fig. 3*A*. The AVP secretory responses of these longer duration stimulations were found to be nearly equivalent in quantity to those of the shorter duration $K^+$ pulses (Fig. 3*B*). The $t_{\frac{1}{2}}$ (half-time, see Methods) of the secretory response was $45 \pm 6$ s ($n = 13$). In contrast, the change in $[Ca^{2+}]_i$ was found to remain elevated throughout the duration of the stimulus. Complete recovery of $[Ca^{2+}]_i$ to prestimulation values on removal of the stimulus frequently required $> 1$ min thereby outlasting the washout of the $K^+$ stimulus (see Methods). It is important to note that relatively slow recoveries of $[Ca^{2+}]_i$ are also observed in response to brief electrical depolarizations under whole-cell voltage clamp. This is illustrated in Fig. 5 where a step depolarization to +10 mV from a holding potential of −90 mV evokes an inward $Ca^{2+}$ current that is accompanied by a significant increase in $[Ca^{2+}]_i$. Despite the brief duration (60 ms) of the inward $Ca^{2+}$ current, the recovery of $[Ca^{2+}]_i$ to a prestimulation value requires *ca* 20 s. The evoked inward current was sensitive to reduction of external $Ca^{2+}$ or to inhibition by $Cd^{2+}$ (data not shown).

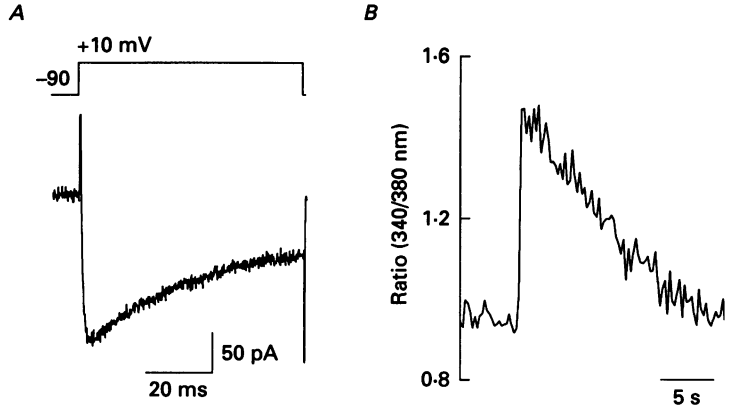


Fig. 5. Comparison of electrically evoked inward $Ca^{2+}$ current and simultaneously monitored change in fura-2 relative fluorescence under whole-cell voltage clamp configuration. *A*, inward $Ca^{2+}$ current was elicited by a 67 ms step to +10 mV from a holding potential of −90 mV. Current shown was not leak subtracted. *B*, change in relative fluorescence ratio of fura-2 recorded in the nerve ending in response to the applied depolarization pulse shown in *A*.

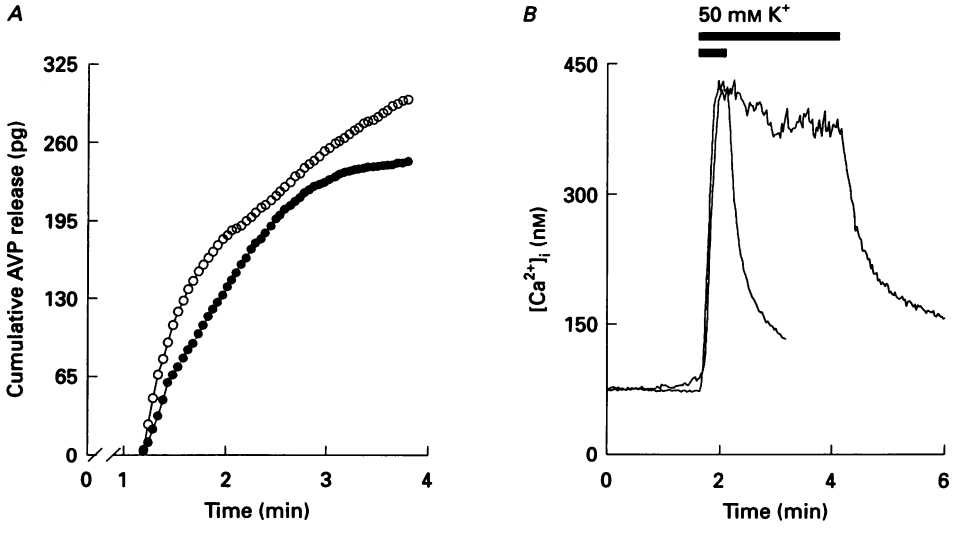


Fig. 6. Effect of duration of $K^+$ depolarization on AVP release and $[Ca^{2+}]_i$. *A*, comparison of a 30 (●) and 150 s (○) 50 mM $[K^+]_o$ depolarization on cumulative AVP release. *B*, representative comparison of the change in $[Ca^{2+}]_i$ induced by 30 and 150 s 50 mM $[K^+]_o$ depolarizations applied sequentially to a single nerve ending. Recovery periods in normal physiological saline occurred between $K^+$ depolarizations with data overlaid for clarity.

The disparity between the secretory responsiveness and the change in $[Ca^{2+}]_i$ to sustained pulses of $K^+$ is shown in Fig. 6. Figure 6*A* shows a representative comparison of cumulative AVP release in response to depolarizing $K^+$ pulses of 30 and 150 s. Both the rates of AVP release and the cumulative amount secreted were, in each case, very similar. This is in contrast to the striking difference in the induced

$[Ca^{2+}]_i$ increase from a single ending sequentially exposed to 30 and 150 s depolarizations (Fig. 6*B*). A slight reduction from evoked peak $[Ca^{2+}]_i$ values was, however, often observed to prolonged 50 mM $K^+$ stimulation. For example, after 90 and 180 s stimulation the $[Ca^{2+}]_i$ was only reduced by an average of $11{\cdot}0 \pm 2{\cdot}6\%$ ($n = 23$) and $28{\cdot}0 \pm 3{\cdot}7\%$ ($n = 13$), respectively.

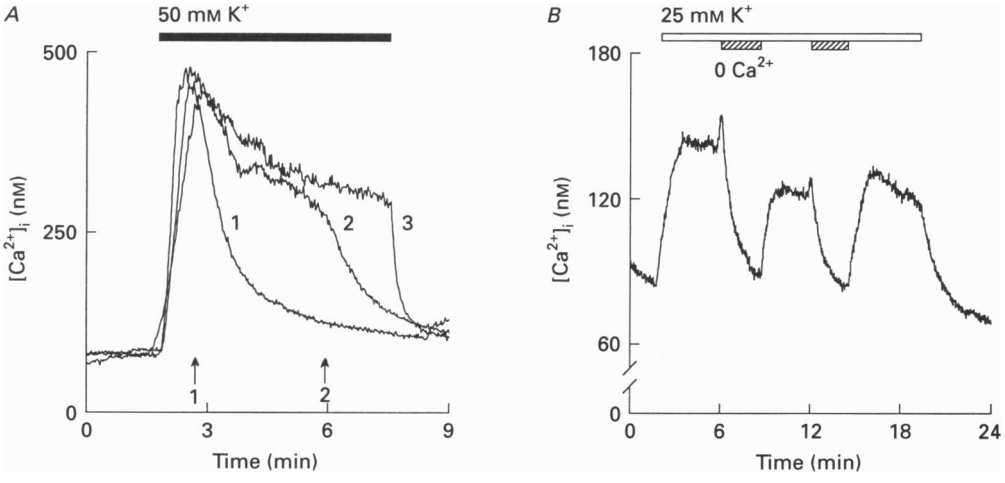


Fig. 7. Susceptibility of the sustained increase in $[Ca^{2+}]_i$ induced by elevated $K^+$ depolarization to a L-type $Ca^2$ channel blocker or to removal of $[Ca^{2+}]_o$. *A*, representative effects of the dihydropyridine nicardipine (10 $\mu$M) applied at 30 (trace 1) or 150 s (trace 2) on $[Ca^{2+}]_i$ following onset of a 50 mM $K^+$ pulse. Control, trace 3. *B*, representative effect of repetitive removal/replacement of $Ca^{2+}$-containing medium on a 25 mM $K^+$-induced $[Ca^{2+}]_i$ increase. Responses similar to those shown in *A* and *B* were observed in six (*A*) and twelve (*B*) nerve endings from three and six preparations, respectively.

To determine if the maintained increase in $[Ca^{2+}]_i$ to a prolonged depolarizing stimulus resulted from sustained $Ca^{2+}$ influx, the effects of a $Ca^{2+}$ channel blocking agent and of reduced calcium media were examined. The dihydropyridine nicardipine is a selective antagonist of L-type $Ca^{2+}$ channels which has previously been shown to inhibit depolarization-induced $Ca^{2+}$ influx and AVP secretion to depolarizing stimuli in a dose-dependent manner (Cazalis *et al.* 1987*a*; Stuenkel, 1990). Application of nicardipine during the plateau phase of a 50 mM $K^+$-evoked $Ca^{2+}$ increase rapidly induced a reversal in $[Ca^{2+}]_i$ towards prestimulatory levels (Fig. 7*A*). The rate of decline of $[Ca^{2+}]_i$ in the presence of nicardipine was 37% ($n = 5$) of the rate of increase in $[Ca^{2+}]_i$ during the onset of the $K^+$ depolarization. Removal of extracellular $Ca^{2+}$ during the evoked $Ca^{2+}$ increase also rapidly resulted in a return in $[Ca^{2+}]_i$ towards resting levels. The $[Ca^{2+}]_i$ recovered to plateau values on readmission of the extracellular $Ca^{2+}$ (Fig. 7*B*). These results combined, suggest that the sustained increase in $[Ca^{2+}]_i$ during $K^+$ depolarization results, in part, from continued $Ca^{2+}$ influx via a dihydropyridine-sensitive $Ca^{2+}$ channel. Concomitant alterations in $Ca^{2+}$ efflux or sequestration have not been examined.

The phasic nature of the secretory response to sustained 50 mM $K^+$ stimulation could result from a rate-limiting reaction of the exocytotic process such as

availability of the exocytotic $Ca^{2+}$-sensitive receptors that promote secretion or of a population of docked, releasable granules. Recovery of secretory responsiveness may then require recovery of $[Ca^{2+}]_i$ to basal conditions in order for the secretory process to be reprimed. To test this hypothesis a series of experiments were performed using

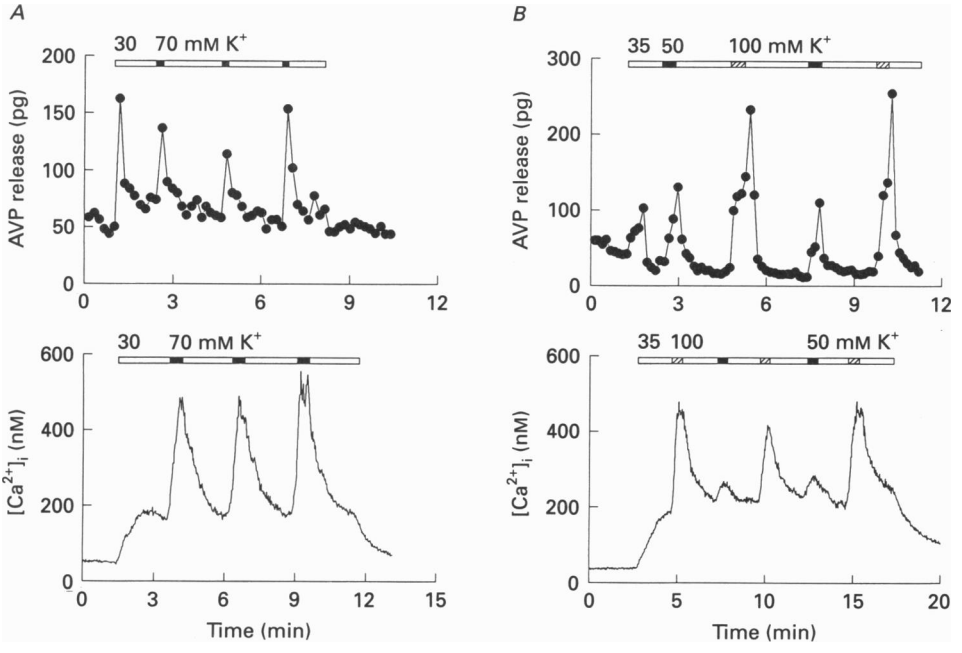


Fig. 8. Effects of sequential alterations in strength of $K^+$ depolarization on AVP release and $[Ca^{2+}]_i$. Alterations in AVP release (upper) and $[Ca^{2+}]_i$ (lower) to sequential applications of 30 and 70 mM $[K^+]_o$ (*A*) or to 35, 50 and 100 mM $[K^+]_o$ (*B*).

steps of $K^+$ depolarization without intervals of recovery between them. Representative results of these experiments are shown in Fig. 8. In these experiments, following the decline in secretion to a sustained step increase in extracellular $K^+$, imposition of a second greater increase in extracellular $K^+$ was capable of reinitiating a phasic secretory response. Note that the $[Ca^{2+}]_i$ changed and remained elevated as a function of the amplitude of the depolarization pulses. Reinitiation of secretion can be repeatedly produced in this manner. Figure 8*B* shows that the size of the secretory response to the second step depolarization was related to the magnitude of the step and correlated closely to the amplitude of the evoked additional increase in intracellular calcium.

To investigate if reactivation of secretion by a further increase in calcium results from different $Ca^{2+}$ sensitivities of the secretory mechanism, a number of $Ca^{2+}$ removal/replacement experiments were performed while keeping the membrane depolarized (Fig. 9). Stimulation of secretion for the usual 30 s (Fig. 9*A*) or for 2 min (Fig. 9*B*) with 50 mM $K^+$ in $Ca^{2+}$-containing media led to the expected decline in AVP secretion. However, subsequent removal of extracellular $Ca^{2+}$ and its replacement led to a resurgent increase in AVP secretion that reached $98 \pm 10\%$ ($n = 3$) of the peak

of the initial response. The $[Ca^{2+}]_i$ closely parallels the secretory response being reduced by chelation of extracellular $Ca^{2+}$ and rebounding on $Ca^{2+}$ replacement (Fig. 9*C*). These results suggest that the phasic nature of the AVP secretory response is not a direct consequence of membrane depolarization nor is it likely to be related to different $Ca^{2+}$ sensitivities of the secretory mechanism.

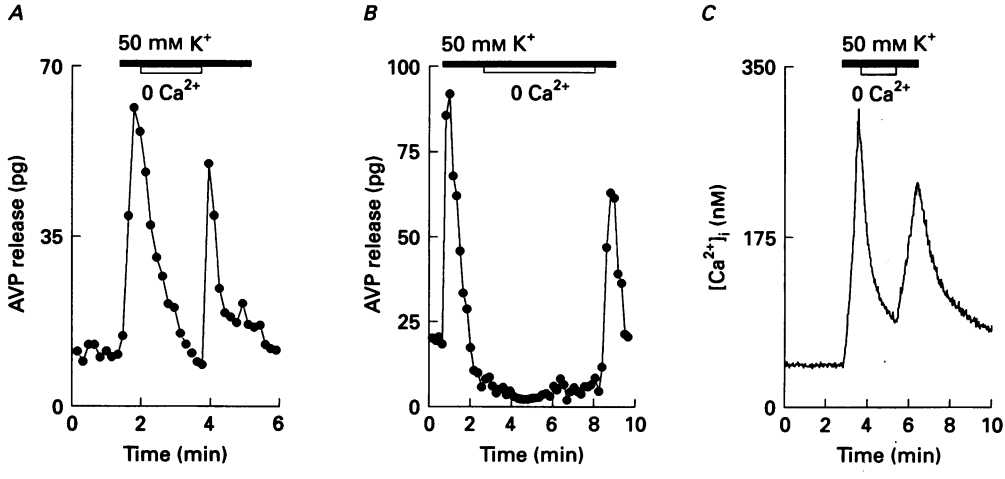


Fig. 9. Effect of external $Ca^{2+}$ removal and replacement on AVP release during sustained 50 mM $K^+$ depolarization. Isolated nerve endings were depolarized with 50 mM $K^+$ for 30 (*A*) or 120 s (*B*) prior to $Ca^{2+}$ removal (no added $Ca^{2+}$ plus 2 mM EGTA). *C*, effects of $Ca^{2+}$ removal/replacement on $[Ca^{2+}]_i$ corresponding to a time frame illustrated in *A*.

*Sensitivity of the secretory mechanism to $Ca^{2+}$*

Membrane depolarization and activation of $Ca^{2+}$ influx via voltage-dependent $Ca^{2+}$ channels generates a gradient of $[Ca^{2+}]_i$ which may reach concentrations of 100 $\mu$M, or greater, at release sites (Roberts, Jacobs & Hudspeth, 1990; Augustine *et al.* 1991). In an effort to assess if the generation of a high local $[Ca^{2+}]$ near the plasma membrane is a necessity for triggering a secretory response, a number of experiments were performed under altered external calcium concentrations ($[Ca^{2+}]_o$). Figure 10 shows the relationship between 50 mM $K^+$-induced increases in $[Ca^{2+}]_i$ and AVP secretion over a range of $[Ca^{2+}]_o$ equivalent to a 1000-fold reduction of the electrochemical gradient for $Ca^{2+}$. A reduction of $[Ca^{2+}]_o$ by 10-fold produced an AVP secretory response to 50 mM $K^+$ depolarization averaging $28{\cdot}6 \pm 1{\cdot}2\%$ ($n = 4$) of that in 2·2 mM $[Ca^{2+}]_o$ (Fig. 10*A*). Surprisingly, as shown in Fig. 9*B*, significant stimulation of AVP secretion could also be observed at $[Ca^{2+}]_o$ as low as 20 $\mu$M ($11{\cdot}2 \pm 2{\cdot}9\%$ of response in 2·2 mM $[Ca^{2+}]_o$; $n = 8$). The increase in secretion at these low $[Ca^{2+}]_o$ correlate to observations of significant 50 mM $K^+$-induced increases in averaged $[Ca^{2+}]_i$ at $[Ca^{2+}]_o$ as low as 5 $\mu$M (Fig. 10*C*).

A quantitative comparison between $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ for both basal and stimulated ($K^+$-evoked) changes, plotted on a semilogarithmic scale, is shown in Fig. 10*D*. With the exception of values at 10 mM $[Ca^{2+}]_o$ basal $[Ca^{2+}]_i$ remains stable over the range of $[Ca^{2+}]_o$ tested. Complete loss of 50 mM $K^+$-induced increases in averaged

$[Ca^{2+}]_i$ were observed at 1 $\mu$M $[Ca^{2+}]_o$. At $[Ca^{2+}]_o$ of 10 mM a consistent decrease ($n = 13$) in depolarization stimulated $[Ca^{2+}]_i$ was observed with respect to values in 2·2 mM $[Ca^{2+}]_o$. The apparent anomaly at 10 mM $[Ca^{2+}]_o$ is paralled by observations of reduced AVP secretion under these conditions (Douglas & Poisner, 1964).

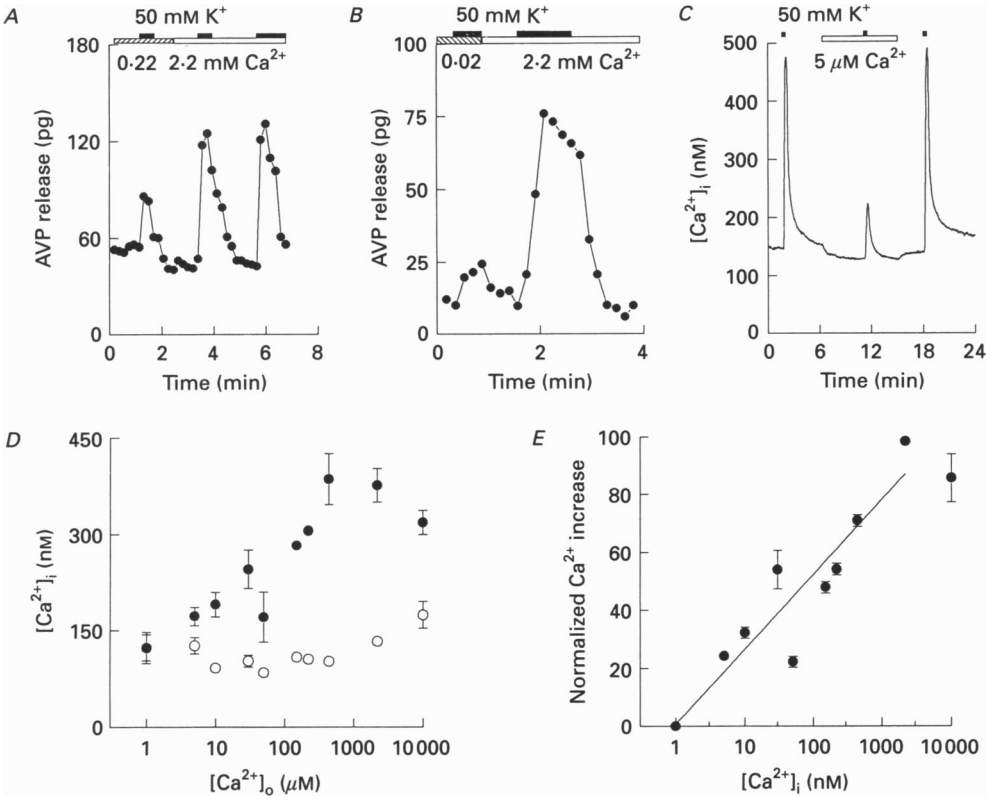


Fig. 10. Effect of $[Ca^{2+}]_o$ on $K^+$ depolarization induced increases in AVP release and $[Ca^{2+}]_i$. *A* and *B*, representative data comparing AVP release induced with 50 mM $K^+$ at 0·22 (*A*) and 0·02 (*B*) mM $[Ca^{2+}]_o$ with that at 2·2 mM $[Ca^{2+}]_o$. *C*, comparison of 50 mM $K^+$-induced increase in $[Ca^{2+}]_i$ under control (2·2 mM) and reduced (5 $\mu$M) $[Ca^{2+}]_o$. *D*, relationship between $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ under resting and 50 mM $K^+$ depolarizing conditions. Data represent mean values ± S.E.M. ($3 < n < 13$). Recovery of full responsiveness to 50 mM $[K^+]_o$ in 2·2 mM $[Ca^{2+}]_o$ was greater than 90% for each condition. *E*, relationship between $[Ca^{2+}]_o$ and normalized $[Ca^{2+}]_i$ induced by 50 mM $K^+$ depolarization.

Replotting the $Ca^{2+}$ data as a normalized quantity, i.e. relative to changes in evoked $[Ca^{2+}]_i$ measured in two 50 mM $K^+$ depolarizations taken prior to the test condition is shown in Fig. 10*E*. An important observation is that secretion could be evoked by 50 mM $K^+$ depolarization at 20 $\mu$M $[Ca^{2+}]_o$ demonstrating that induction of secretion is not dependent upon generation of locally high $[Ca^{2+}]_i$ in the range of hundreds of micromolar near the plasma membrane. It should be noted that we have found no evidence for caffeine (10 mM) or, by electrophysiological analysis, of $Ca^{2+}$-sensitive intraterminal $Ca^{2+}$ stores in these nerve endings.

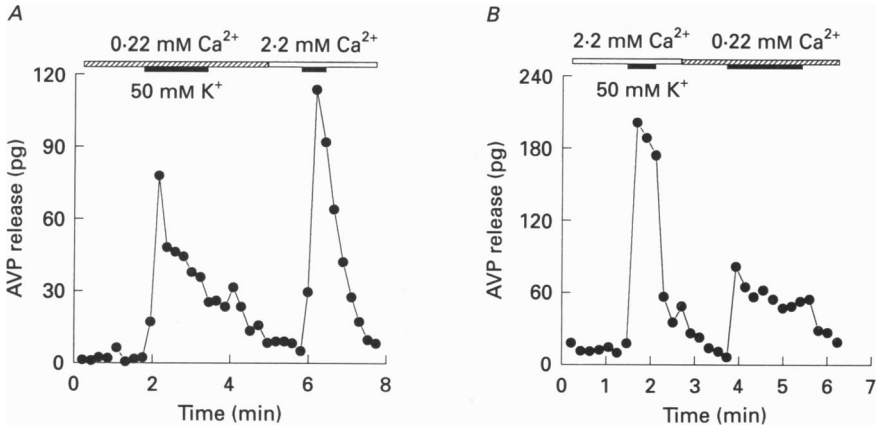


Fig. 11. Effect of reduced $[Ca^{2+}]_o$ on the time course of AVP release. Comparison of $K^+$-induced (50 mM) AVP release in 0·22 mM $[Ca^{2+}]_o$ with release induced in 2·2 mM $[Ca^{2+}]_o$ (*A*) or in reverse order of external $[Ca^{2+}]$ (*B*).

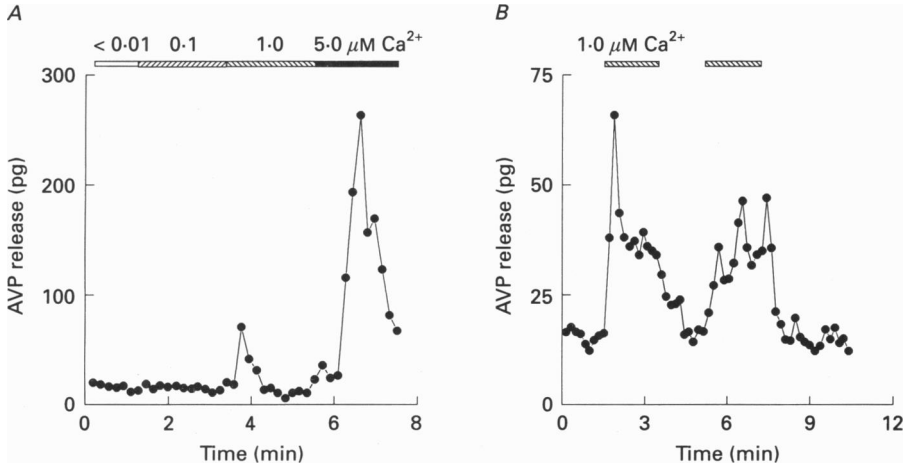


Fig. 12. Effect of $Ca^{2+}$ application on AVP secretion in permeabilized nerve endings. *A*, the effect of sequential step increases in $[Ca^{2+}]$ on AVP release from streptolysin-O permeabilized nerve endings. $[Ca^{2+}]$ (μM) as indicated by the bar above the plot. *B*, effect on AVP release in digitonin-permeabilized nerve endings of step increases to 1 μM $Ca^{2+}$, with intervening period in $Ca^{2+}$-free medium.

A series of experiments were designed to determine if the reduction of the electrochemical $Ca^{2+}$ gradient across the membrane affected the temporal nature of the secretory response. Figure 11 shows representative examples of 50 mM $K^+$-stimulated secretion to a 10-fold reduction in the $Ca^{2+}$ gradient. In contrast to the phasic secretory response observed in 2·2 mM $[Ca^{2+}]_o$, secretion of AVP at 0·22 mM $[Ca^{2+}]_o$ was more sustained ($t_{\frac{1}{2}} = 118 \pm 12$ s, $n = 7$). The ability of the depolarizing stimulus to sustain secretion at lowered $[Ca^{2+}]_o$ was observed even in those preparations which had received an initial stimulation period in 2·2 mM $[Ca^{2+}]_o$ (Fig. 11*B*).

*Time course of secretion in permeabilized nerve endings*

The above information led us to investigate if the rapid phasic characteristic of AVP secretion is retained in permeabilized nerve endings. They were exposed to fast-flow perfusion conditions where direct access of $Ca^{2+}$ to the cytoplasm is provided and $[Ca^{2+}]_i$ concentration gradients are avoided. Nerve endings were permeabilized with digitonin or streptolysin-O in nominally $Ca^{2+}$-free medium followed by $Ca^{2+}$ replacement. The effects of such treatments on the time course of AVP secretion are shown in Fig. 12. Perfusion of the nerve endings with 1 $\mu$M $[Ca^{2+}]_o$ results in a phasic secretory response which, when followed by exposure to 5 $\mu$M $[Ca^{2+}]_o$ evokes a second secretory response. The results demonstrate that even when held at a constant, uniform, $[Ca^{2+}]$ the nerve endings show a rapid phasic secretory response. To investigate whether the phasic release response in the permeabilized nerve endings can be explained by different $Ca^{2+}$ sensitivities of the secretory mechanism, a number of experiments were performed using repetitive stimulation at the same $[Ca^{2+}]$. The results of these experiments are shown in Fig. 12*B*. Here repetitive stimulation with medium containing 1 $\mu$M $[Ca^{2+}]$ separated by periods in nominally $Ca^{2+}$-free medium is able to repetitively evoke AVP release.

## DISCUSSION

We have found, in isolated neurohypophysial nerve endings, that there is a clear dichotomy between the time course of vasopressin secretion, quantified on the time scale of seconds, and that of spatially averaged $[Ca^{2+}]_i$ to sustained membrane depolarization. This was evidenced by a secretory response that was rapid in onset but brief in duration ($t_{\frac{1}{2}} = 45 \pm 6$ s) while the evoked increase in spatially averaged $[Ca^{2+}]_i$ exceeded the duration of the stimulus even in nerve endings depolarized for a period of 65 ms. The secretory time course reported here is markedly similar to that reported, recently, from monitoring of changes in membrane capacitance at the level of single neurohypophysial nerve endings under voltage clamp conditions Lindau *et al.* 1992). The close correspondence occurs despite differences in secretory stimuli (elevated $[K^+]$ *versus* electrical depolarization) and in monitoring of release from single *versus* populations of nerve endings. Our findings suggest that the transient nature of the secretory response is not likely to be a direct result of a decline in the $Ca^{2+}$ conductance of the nerve ending alone or of depletion of granules available for release. Rather, in addition to contributions from the components above, the rate and extent of secretion may be regulated, not only by the absolute increase in $[Ca^{2+}]_i$ but also, following a given time interval, by changes in $[Ca^{2+}]_i$.

*The time course of vasopressin secretion*

Several forms of use-dependent plasticity can occur within synaptic sites to generate increased levels or durations of chemical messenger release (Zucker, 1989). For the neurohypophysis, secretion is a sensitive function of the impulse patterning and frequency (Cazalis *et al.* 1985). Neurohypophysial action potentials broaden with increasing frequency (Gainer, Wolfe, Obaid & Salzberg, 1986; Bourque, 1990) and result in enhanced $Ca^{2+}$ influx (Jackson, Konnerth & Augustine, 1991). Similarly, patterning of impulses into bursts has been shown to provide an augmentation over

tonic impulse firing in the rise in $[Ca^{2+}]_i$ (Brethes, Dayanithi, Letellier & Nordmann, 1987). However, in response to a constant sustained membrane depolarization, vasopressin release (Muller, Thorn & Torp-Pedersen, 1975; Nordmann, 1976), as well as neurotransmitter and neurohormone release from a variety of systems (Baker & Rink, 1975; García *et al.* 1976; Birman & Meunier, 1985), declines prior to removal of the stimulus. This has largely been interpreted as resulting from a decline or inactivation of $Ca^{2+}$ conductance in the secretory element (Baker & Rink, 1975; Nordmann, 1976; Garcia *et al.* 1976; Datyner & Gage, 1980; Holz, Senter & Frye, 1982; Birman & Meunier, 1985). The inactivation hypothesis is consistent with a large body of evidence demonstrating voltage- and $Ca^{2+}$-dependent inactivation properties of $Ca^{2+}$ channels (Hagiwara & Byerly, 1981; Tsien, 1983) including evidence from recent whole-cell and single channel $Ca^{2+}$ currents in isolated neurohypophysial nerve endings (Lemos & Nowycky, 1989). A number of results of the present study, however, suggest that $Ca^{2+}$ channel inactivation alone is inadequate to explain the transient nature of the AVP secretory response to sustained stimulation. These include: (1) the presence of continued $Ca^{2+}$ influx through a dihydropyridine-sensitive mechanism throughout the period of sustained stimulation, (2) the finding of similar transient secretory responses in permeabilized nerve endings when challenged with continuously elevated $[Ca^{2+}]$, (3) the ineffective block of $Ca^{2+}$ influx on readdition of $Ca^{2+}$ following a period of depolarization in the absence of $Ca^{2+}$ and (4) the reinitiation of secretion following its decline by directly increasing the level of $K^+$ depolarization. On the other hand $Ca^{2+}$ channel inactivation in the neurohypophysial nerve endings can be observed even with short depolarizing pulses (Fig. 6; see also Lemos & Nowycky, 1989) and cannot be entirely excluded as influencing the time course of the secretory response. Indeed, in the presence of the $Ca^{2+}$ channel agonist Bay K 8644 a small but significant increase in AVP release is observed (Cazalis *et al.* 1987*a*). Moreover, the slower decline of AVP secretion in 0·22 mM external calcium may result from a smaller $Ca^{2+}$-dependent inactivation of voltage-dependent $Ca^{2+}$ channels or less activation of downstream regulators of AVP secretion.

The existence of close spatial relationships between the sites of $Ca^{2+}$ influx and the sites of exocytosis has been frequently postulated based on morphological evidence (Pumplin *et al.* 1981) and on the short delay between the activation of $Ca^{2+}$ current and the onset of transmitter release (Katz & Miledi, 1965; Miledi & Slater, 1966; Llinás *et al.* 1976; Almers, 1990). Clearly, one complication to the interpretation of the findings in the present study is that the spatially averaged $[Ca^{2+}]_i$ measurements are poorly representative of the dynamics of local $[Ca^{2+}]$ at the release site. In a spatially focused model, impulse-mediated depolarization rapidly generates highly localized $Ca^{2+}$ domains at release sites while rapid binding to receptor sites and diffusion from release sites limit the duration of the secretory event (Miledi & Parker, 1981). Additional complexity is added when considering evidence suggesting the release process is a power function of the concentration of a $Ca^{2+}$-receptor complex (Dodge & Rahamimoff, 1967; Lester, 1970; Smith, Augustine & Charlton, 1985), and that $Ca^{2+}$ channels may be clustered on the membrane surface (Augustine *et al.* 1985; Roberts *et al.* 1990). In the present study we observed that secretion, following its decline to a maintained $[K^+]_o$ pulse, can be reinitiated by a further step increase in

$[K^+]_o$. This can be interpreted with regard to the above model as resulting from a time-dependent dissolution of the initially developed $Ca^{2+}$ gradient, which is transiently re-established on further depolarization and activation of $Ca^{2+}$ influx (Fig. 8). The transient establishment of locally high $[Ca^{2+}]_i$ may be necessary for activation of the chain of events regulating secretion. Similarly, the reinitiation of AVP release in the face of continued depolarization on readmission of $[Ca^{2+}]_o$ may simply allow re-establishment of steep intracellular $Ca^{2+}$ gradients (Fig. 9). On the other hand, the observation that similar transient AVP secretory responses occur in permeabilized nerve endings to a given sustained $Ca^{2+}$ concentration argues against the time course of secretion being entirely dependent upon the rapid dissipation of highly localized $[Ca^{2+}]_i$ domains. The permeabilized nerve ending preparation provides direct access of $Ca^{2+}$ to the cytoplasm thereby eliminating the generation of local $Ca^{2+}$ domains and of $Ca^{2+}$ concentration gradients. Reinitiation of secretion by raising $[Ca^{2+}]_i$ further or by removal of $Ca^{2+}$-containing medium followed by $Ca^{2+}$ replacement (Fig. 12) demonstrates that the phasic secretory response in permeabilized nerve endings is not the result of loss of secretory responsiveness and complicates explanations of the transient secretory responses on $Ca^{2+}$-dependent feedback inhibition of $Ca^{2+}$ entry or exocytosis.

A number of additional possibilities could participate in regulating the time course of AVP secretion to maintained depolarization. For example, the secretory time course could be related to depletion of a readily releasable pool of predocked secretory granules which then must be refilled by mobilization of granules from an adjacent or interior site within the nerve ending (Schweizer *et al.* 1989). This possibility has been repeatedly discussed and discounted for a number of cell types based on the ability to re-evoke secretion by addition of $Ca^{2+}$ ionophores (Nordmann, 1976; Birman & Meunier, 1985) or by determining that the percentage of product secreted represented a small percentage of the total product present (Nordmann, 1976). In the present study, evidence that the decline in AVP secretion is not the result of depletion of a releasable pool is supported by the ability to reinitiate secretion on application of an additional step depolarizing stimulus. This finding also argues against a situation where release declines as a direct result of reaching the capacity of the vesicle receptor sites on the plasma membrane. On the other hand, the ability to re-evoke secretion in this manner might be interpreted as evidence that the secretory machinery exhibits different and fixed $Ca^{2+}$ sensitivity thresholds leading to a *quantal* nature of the secretory response similar to that proposed for $Ca^{2+}$ release from inositol 1,4,5-trisphosphate-sensitive internal stores (Irvine, 1990). However, the results of the $Ca^{2+}$ removal/re-addition experiments on intact (Fig. 9) and on permeabilized nerve endings (Fig. 12) argue against such a hypothesis as does a previous report on this preparation (Cazalis, Dayanithi & Nordmann, 1987*b*) and on permeabilized chromaffin cells (Knight & Baker, 1982). Nevertheless, it should be noted that evidence exists for multiple releasable granule pools in chromaffin cells with distinct secretory rates, based on direct monitoring of changes in membrane capacitance (Bookman & Schweizer, 1988). This appears to be the case for isolated neurohypophysial nerve endings as well, which show an initial, rapid (millisecond scale) rate followed by a slower rate of membrane capacitance increase to an applied step depolarization (Lindau *et al.* 1992).

The reactivation of AVP secretion, following its initial decline by treatments which induce an increasing change in $[Ca^{2+}]_i$, is similar to characteristics of catecholamine release reported on electropermeabilized chromaffin cells by Knight & Baker (1982). A hypothesis they present is, with modification, consistent with many of the findings of the present report. The hypothesis of Knight & Baker (1982) states that the probability of an exocytotic fusion event is related to the rate at which a change occurs in an unknown intermediate at the release site. Thus, release only occurs while a change in the intermediate is occurring and not to a steady increased level of the intermediate. For the neurohypophysial nerve endings increased AVP release evoked by an increased $[Ca^{2+}]_i$ continues only for a brief period ($t_{\frac{1}{2}} = 45$ s). Beyond this time period a steady, but significantly elevated $[Ca^{2+}]_i$ is unable to support AVP release although a change in $[Ca^{2+}]_i$ can reinitiate secretion. We suggest, therefore, that in addition to the absolute $[Ca^{2+}]_i$ at the release site, periodic changes in $[Ca^{2+}]_i$ are important to maintain exocytotic activity. Unlike the hypothesis of Knight & Baker (1982), however, which suggests the necessity for a continuously changing intermediate, our results suggest continued secretion would require only periodic changes in an intermediate. In this manner, the $Ca^{2+}$ signal controlling secretion resembles, in part, the periodic oscillations in $[Ca^{2+}]_i$ observed in non-excitable cells to activation of $Ca^{2+}$ mobilizing receptors. A similar conclusion has recently been reported from studies on AVP release from permeabilized nerve endings (Cazalis *et al.* 1987*b*) and on prolactin release (Sato, Wang & Greer, 1990).

*$Ca^{2+}$ sensitivity for exocytosis*

Current $Ca^{2+}$-sensing methodology does not allow changes in $[Ca^{2+}]_i$ to be quantified within the time frame of a single exocytotic secretory event and spatially resolved over distances such as occur between a $Ca^{2+}$ channel and release site. The dynamics and amplitude of $[Ca^{2+}]_i$ changes within these temporal and spatial domains have, thus, largely been relegated to mathematical modelling strategies (Stockbridge & Moore, 1984; Simon & Llinás, 1985; Fogelson & Zucker, 1985). As a result, the $[Ca^{2+}]_i$ at the release site that is necessary to initiate exocytosis in intact cells remains unknown. The findings of the present report suggest that the concentration necessary at the vasopressin release site is approximately 20 $\mu$M or possibly less. This estimate is based on the ability to evoke secretion in medium containing 20 $\mu$M free $[Ca^{2+}]$. The actual $[Ca^{2+}]_i$ requirement may be lower when placed in the context of recent experimental predictions of 1:10 to 1:20 ratios of $[Ca^{2+}]_o/[Ca^{2+}]_i$ (Roberts *et al.* 1990; Augustine *et al.* 1991). These predictions would agree with a free $[Ca^{2+}]$ of the order of 1–10 $\mu$M at which secretion can be readily observed in a variety of permeabilized cells. Digitonin-permeabilized neurohypophysial nerve endings likewise show a half-maximal release at 1·7 $\mu$M $[Ca^{2+}]$ (Cazalis *et al.* 1987*b*). In contrast, recent experimental findings in intact cells, using exogenously introduced $Ca^{2+}$ buffers (Augustine *et al.* 1991) or by monitoring activity of $Ca^{2+}$-sensitive ion channels as indicators of $[Ca^{2+}]_i$ near the membrane (Roberts *et al.* 1990), have suggested that local $[Ca^{2+}]_i$ reaches tens to hundreds of micromolar in response to $Ca^{2+}$ channel activation. The significance to generation of a $[Ca^{2+}]_i$ greater than that necessary for induction of secretion may be related to the rate of release, as recently shown in chromaffin cells (Augustine & Neher, 1992).

*Physiological relevance of transient secretory responses*

In conclusion, the results of the present paper strongly suggest that evoked AVP release from neurohypophysial nerve endings is not only dependent on the absolute $[Ca^{2+}]_i$ but also upon periodic changes in intraterminal $[Ca^{2+}]$. These findings could largely explain the potentiating effect of impulse bursting activity on neuropeptide secretion observed *in vivo* upon hormone demand (Cazalis *et al.* 1985). The bursting activity, characteristic of the magnocellular neurones (for review see Poulain & Wakerley, 1982), could promote an increase in $[Ca^{2+}]_i$ whereas, the silent interval periods between the bursts would allow the nerve endings to recover their basal $[Ca^{2+}]_i$. In this manner the $[Ca^{2+}]_i$ would not only reach the threshold value for release but would fulfil the conditions necessary for continued tonic neurotransmitter/neurohormone release.

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