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HORMONE RELEASE FROM ISOLATED NERVE ENDINGS OF THE RAT NEUROHYPOPHYSIS

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SUMMARY

1. Isolated neurosecretory nerve endings were prepared from rat neurohypophyses. The amount of vasopressin (AVP) and oxytocin released was measured by radioimmunoassay.

2. The amount of hormone release under resting conditions was not affected by external calcium (Ca_o^{2+}). Secretion decreased by *ca.* 50% when external sodium (Na_o^+) was replaced by choline or sucrose.

3. Ouabain did not modify the basal AVP release.

4. The Na^+ ionophore monensin increased the release of AVP only in the presence of Na_o^+. This increase was maintained during prolonged exposure to the ionophore and occurred in the presence of Ca_o^{2+} only.

5. In the presence of Ca_o^{2+}, the amount of evoked hormone release was dependent on the external K^+ concentration. Half-maximal activation was achieved with *ca.* 40 mM-K^+. The K^+-induced secretion was potentiated in Na^+-free solution.

6. Prolonged 100 mM-K^+-induced depolarization in the presence of Ca_o^{2+} gave rise to a large increase in hormone secretion which decreased with time ($t_{\frac{1}{2}} = 2{\cdot}5$ min). The release could be reactivated after permeabilization of the nerve terminals in the presence of micromolar concentrations of Ca^{2+}.

7. A stepwise paradigm in which K_o^+ is incrementally increased to 25, 50, 75 and then 100 mM released more AVP than a prolonged exposure to 100 mM-K^+.

8. Veratridine increased the amount of AVP released. This effect was considerably reduced in the absence of Na_o^+ and abolished in the presence of D600.

9. The depolarization-induced AVP release was blocked by different Ca^{2+}-antagonists. Their effectiveness was nitrendipine = nicardipine > Cd^{2+} > Gd^{3+} > Co^{2+} = Mn^{2+}.

10. The dihydropyridine Bay K 8644 potentiated both the basal and the K^+-evoked AVP release. Its maximal effect was obtained with 25–50 mM-K_o^+.

11. In conclusion, the isolated neurohypophysial terminals which have both Na^+ and Ca^{2+} channels and release AVP and oxytocin upon depolarization might be an excellent system to study further the mechanisms leading to secretion of neurohormones.

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INTRODUCTION

The concept of stimulus–secretion coupling elaborated two decades ago by Douglas & Rubin (1963), Douglas & Poisner (1964*a*, *b*) and Douglas (1968) implies that following depolarization of the plasma membrane there is an entry of Ca^{2+} ions which triggers the release of neurotransmitters, neurohormones or any substance packed into secretory granules or vesicles. The relationship between the entry of Ca^{2+} and the mechanism of release has been studied in a large variety of tissues. One of the main problems in studying release mechanisms is to find a system in which the steps leading to secretion can be separated from those of synthesis and transport. The hypothalamo–neurohypophysial system offers the advantage of studying the process of neurosecretion at different levels. The anatomical separation of the hypothalamic cell bodies from the nerve terminals, located in the neurohypophysis, allows the analysis of the coupling between the electrical activity of the neurones and the peptide release at the level of the nerve endings. Furthermore, because no synthesis of vasopressin and oxytocin occurs in the nerve terminals it is possible to study the packaging, transport and storage of neurohormones at the distinct site of the neurones (for a review, see Morris, Nordmann & Dyball, 1978). Douglas & Poisner (1964*a*, *b*) have shown that depolarization of the rat neural lobe *in vitro* gives rise to the entry of $^{45}Ca^{2+}$ and to vasopressin (arginine vasopressin; AVP) release. Furthermore, it was in the neurohypophysis that it was first shown that a Ca^{2+} channel similar to that found in the squid axon (Baker, Meves & Ridgway, 1973) is coupled to the mechanism of neurosecretion (Dreifuss, Grau & Nordmann, 1973). However, like any other system, the neurohypophysis *in toto* suffers from disadvantages. For instance, the nerve endings are surrounded by pituicytes which might modulate and mask some intrinsic properties of the nerve endings. Furthermore, the tortuous extracellular space and the basal membrane prevent the diffusion of large molecules which could be used as probes. In the present paper we describe some of the properties of isolated neurohypophysial nerve endings which circumvent these problems. It is shown that these isolated nerve terminals or 'neurosecretosomes' (La Bella & Sanwal, 1965) respond to depolarization and that hormone release is Ca^{2+} dependent. It is concluded that this new preparation which, in contrast to the classical brain synaptosomes, contains well-defined secretory material, might be a useful system to further study the stimulus–secretion coupling mechanisms. In the following paper (Cazalis, Dayanithi & Nordmann, 1987), using a procedure which bypasses the depolarization step, we analyse more specifically the requirements for exocytosis.

METHODS

Neurointermediate lobes were isolated from male rats (Wistar) weighing 250–300 g and washed with normal Locke solution (see below) for a period of 3–5 min. The medium was then replaced by a solution containing 270 mM-sucrose, 0·01 mM-EGTA (ethyleneglycol-bis (β-aminoethylether)*N*-*N*′-tetracetic acid) and 10 mM-HEPES, pH 7·2. The neurohypophyses were then transferred to a 1 ml glass–Teflon homogenizer containing 1 ml sucrose medium and homogenized gently with five strokes. The resulting homogenate was centrifuged at 600 *g* for 4 min in a Sorval RC-5B centrifuge using an HB-4 type rotor with adapters for 1·5 ml plastic Eppendorf tubes. The resulting supernatant was centrifuged at 3400 *g* for 15 min and the pellet, which contained mostly isolated nerve endings and swellings as checked by electron microscopy (J. J. Nordmann & F. D. Shaw, unpub-

lished), was resuspended in normal Locke solution of the following composition (mM): NaCl, 140; KCl, 5; $NaHCO_3$, 5; $CaCl_2$, 2·2; $MgCl_2$, 1; glucose, 10; HEPES, 10; 0·01% bovine serum albumin; pH 7·2. The nerve endings were loaded by means of a 1 ml plastic syringe on to a 0·22 μm Millipore filter in a small plastic perfusion chamber with an internal volume of 50 μl. A pre-filter (Millipore, type AP 25) was placed onto the 0·22 μm filter before loading of the material. This prevents the 0·22 μm filter from becoming blocked during the course of the perfusion. From the time of isolation of the neural lobe to that of perfusion we tried to maintain the temperature of the solutions as near to 37 °C as possible. For instance, the refrigeration of the centrifuge was switched off and the rotor and the adaptors were pre-warmed by centrifugation at 15000 rev/min for 10 min. Thus the temperature was 32–36 °C. Furthermore, the perfusion chamber and the syringe used to load the nerve endings were pre-warmed at 37 °C. After loading, the plastic chamber was placed in an incubator (Prolabo) thermoregulated to 37·0 ± 0·2 °C. The isolated nerve endings were perfused by means of a peristaltic pump (Minipuls 2, Gilson) for 35 min at a flow rate of 50 μl/min which was then increased to 100 μl/min. Collection of the perfusate by means of a fraction collector (Isco Model) started 45–60 min after loading. Except where stated otherwise the fractions were collected during 2 min, diluted with 10 mM-HEPES, pH 6·8, by a factor of 5–20 depending upon the experiments and were immediately frozen until the hormonal assay. At the end of each experiment the filters were placed in 1 ml 0·5% (w/v) Triton X-100 and frozen. After thawing, the material was sonicated, centrifuged for 10 s at 9000 *g* on a Beckman microfuge and the AVP and oxytocin content of the supernatant was measured by radioimmunoassay (Cazalis, Dayanithi & Nordmann, 1985). The evoked hormone release was calculated by subtracting the mean basal release determined in the first five fractions preceding the onset of the stimulus from the amount of hormone found in each fraction.

The neurosecretosome preparation is surprisingly pure according to some preliminary morphological observations (J. J. Nordmann, unpublished). Briefly, the crude homogenate contains mostly nerve endings and nerve swellings. The later are storage sites and do not contain microvesicles (see Morris, 1976; Nordmann, 1977). It was surprising to find that very few, if any, pituicytes (glial cells) resist homogenization. Some of the endings or swellings have diameter of up to 7 μm although, as observed *in situ* (Nordmann, 1977), most of the endings have a diameter of about 1–2 μm. Most of the spherical elements of the crude homogenate showed positive immunocytochemical reaction with an antiserum against total neurophysins (J. J. Nordmann, unpublished). The 600 *g* centrifugation seems to eliminate the large nerve terminals and thus the 3400 *g* pellet used in the present study represents mostly nerve endings. As observed with nerve endings prepared from the crab sinus gland (Nordmann, Weatherby & Haylett, 1986), some neurosecretosomes are exposed to the extracellular medium during homogenization as judged by the presence in the terminals of added Lucifer Yellow.

The results are given as mean ± standard error of the mean, the number of experiments being in parentheses. Statistical differences were analysed by the Student's *t* test.

RESULTS

Basal release of hormone from isolated nerve endings

In a preliminary study (unpublished) we have found that, after loading the endings in the perfusion chamber, AVP and oxytoxin release occurs in two phases. The first could be described by a single exponential with a fast decay and is presumably associated with the loss of hormone from damaged nerve endings. Basal release of AVP and oxytoxin could be considered as stabilized 40–60 min after loading. Therefore, in all the subsequent experiments the neurosecretosomes were perfused with physiological saline for a period of 45–60 min and the hormone released was collected after this initial period of time. In one series of experiments, the neurosecretosomes were perfused with 2 μM-Ca^{2+}-containing Locke solution for a period of 16 min. The external Ca^{2+} concentration $[Ca^{2+}]_o$ was then raised for periods of 15 min to 1 mM and subsequently to 2 mM. No detectable increase in AVP release was observed

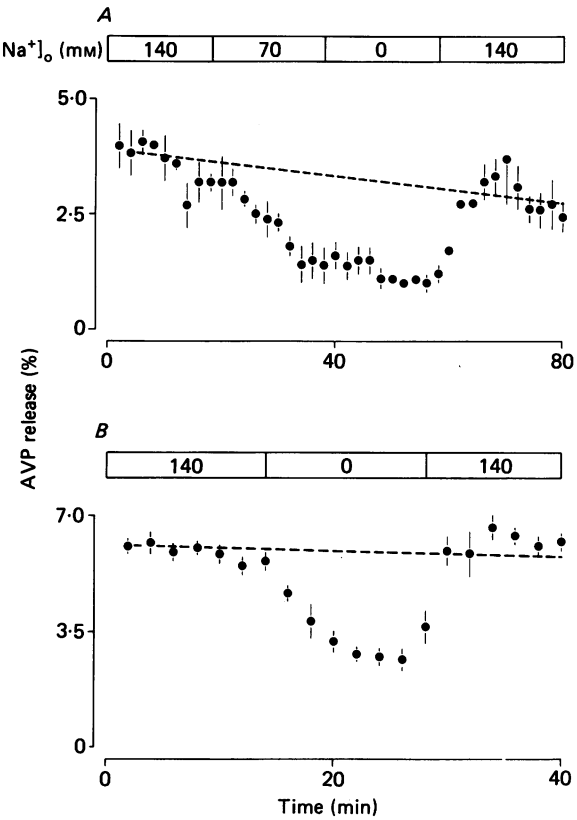

Fig. 1. The effect of Na_o^+ on basal AVP release from isolated neurosecretory nerve endings. Collection of fractions started 60 min after perfusion with medium containing 140 mM-Na^+. The Na_o^+ concentration was as indicated at the top of each panel. The osmolarity of the perfusate was maintained with choline chloride (*A*) or sucrose (*B*). The results are expressed as a percentage of the total AVP released during 40 min of perfusion. They are given as means ± S.E. of means ($4 \leqslant n \leqslant 6$). Only the standard errors of the mean larger than the symbols used are given. The dashed line represents the expected basal release during continuous perfusion with saline containing 140 mM-Na^+.

under these conditions, suggesting that the $[Ca^{2+}]_o$ has no detectable influence on the basal release *in vitro*. Fig. 1 shows the effects of varying the external Na^+ concentration ($[Na^+]_o$) on the basal release of AVP from isolated nerve endings. A reduction of Na_o^+ gave rise to a significant diminution of the AVP release and this was observed both when sodium chloride was replaced either with an ionic (choline chloride, Fig. 1*A*) or non-ionic (sucrose, Fig. 1*B*) substance.

In a series of experiments the nerve endings were perfused for a period of 2 h with normal Locke solution containing 10^{-5} M-ouabain. This cardiac glycoside, which is known to increase the internal Na^+ concentration ($[Na^+]_i$), did not modify the amount of AVP released from the neurosecretosomes.

The role of Na^+ on the basal hormone release was also studied using the

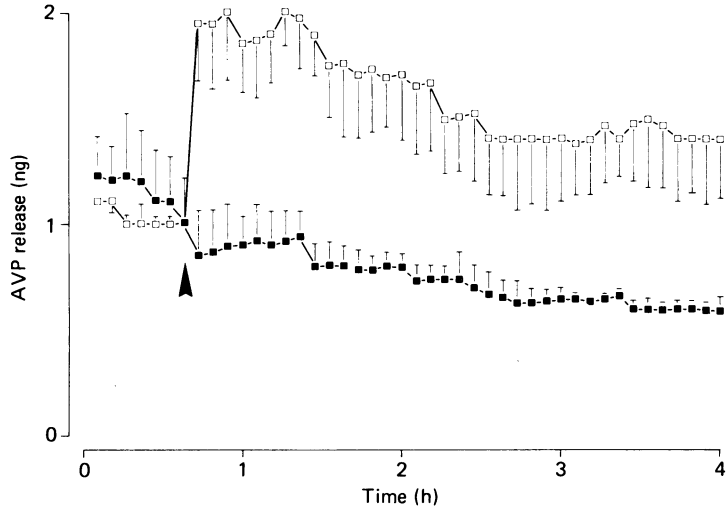

Fig. 2. The effect of the Na^+ ionophore monensin on AVP release from isolated neurosecretory nerve endings. The preparation was perfused either in the presence (□) or absence (■) of 140 mM-Na^+. Monensin (10^{-5} M) was added as indicated by the arrow. The results are expressed as amount of AVP released per 5 min fraction period and are given as means ± S.E. of means ($n = 4$).

Na^+-selective carboxylic ionophore monensin (Pressman, 1976). Isolated nerve endings were perfused with normal Locke solution and 10 μM-monensin was then added. The results are presented in Fig. 2. The ionophore induced a small but maintained increase in AVP release. This is of importance, for it is the first time, to our knowledge, besides the experiments in which a neural lobe was electrically stimulated at a low mean frequency (Cazalis *et al.* 1985), that a stimulus induced a prolonged and sustained hormone release from neurosecretory nerve endings. The amount of hormone secreted during exposure to monensin for 200 min was 30·9 ± 7·7 ng AVP ($n = 4$), which corresponds to about 36% of the total AVP content of the nerve endings. At a concentration of 10^{-6} M, monensin was ten times less potent than at 10^{-5} M. Furthermore the effect of the ionophore was abolished in Na^+-free medium and required the presence of Ca_o^{2+}.

Effect of depolarization on neurohormone release

In order to see if, after isolation, the nerve endings could still release neurohormones as a result of depolarization, the preparation was perfused with Na^+-free, choline-containing medium. K^+ was added for a period of 10 min at different concentrations, choline being reduced accordingly. Fig. 3 illustrates the effect of K_o^+ on the evoked AVP release calculated as described in the Methods. A highly significant increase of hormone release could already be observed with depolarizations induced by 25 mM-K^+ and maximal secretion was observed at 100 mM-K^+. The amount of AVP released upon depolarization with 100 mM-K^+ (9·2 ± 0·7 ng, $n = 8$) corresponded to 10·2 ± 0·6% of the nerve terminal hormonal content. Similarly, exposure to 100

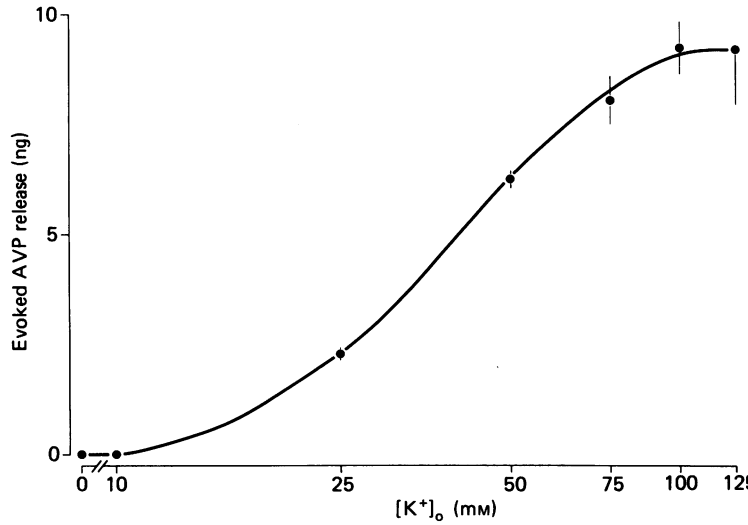

Fig. 3. The effect of $[K^+]_o$ on AVP release from isolated neurosecretory nerve endings. The preparation was perfused with Na^+-free, choline-containing saline. K^+ was increased for a period of 10 min at the concentrations indicated on the abscissa, choline being reduced accordingly. The evoked release was calculated as described in the Methods. The results are given as means ± S.E. of means ($4 \leqslant n \leqslant 8$).

mM-K^+ gave rise to the release of 35·7 ± 1·5 ng oxytocin ($n = 7$), corresponding to 8·0 ± 0·7% of the nerve terminal content. The time courses of AVP and oxytocin secretion were very similar. It has to be pointed out that the AVP and oxytocin content of the preparation was very similar in all the preparations. In another series of experiments the nerve endings were depolarized for 10 min with 100 mM-K^+ in the presence of 40 mM-Na_o^+. The amount of AVP released (6·5 ± 0·6 ng, $n = 6$) corresponded to 70% of that observed when choline was substituted for Na_o^+ (9·2 ± 0·6 ng, $n = 8$). Thus, as already shown *in situ* (Dreifuss, Grau & Bianchi, 1971), Na_o^+ exerts an inhibitory effect on the K_o^+-induced neurohormone release.

Veratridine, which promotes hormone release from isolated neural lobe (Nordmann & Dyball, 1978) induced AVP secretion from isolated neurosecretory nerve terminals. At a concentration of 60 μM the alkaloid evoked, during a period of 10 min, the release of 11·8 ± 0·4 ng AVP ($n = 8$). This effect was reduced by more than 80% (2·3 ± 0·2 ng, $n = 3$) in Na^+-free saline and was abolished in the presence of 100 μM-D600.

Under prolonged K_o^+-induced stimulation of the neurohypophysis the time course of hormone release shows that, after an initial increase, secretion decreases with time even though the stimulus is maintained. This is illustrated in Fig. 4. The neurosecretosomes were perfused at a rate of 360 μl/min and the fractions were collected every 15 s. Increasing the $[K^+]_o$ to 100 mM gave rise to a large release which took about 60 s to reach its maximum. The rate of release was not maintained and decreased rapidly ($t_{\frac{1}{2}} = 2·5$ min). It has been suggested that the observed decrease of AVP release could be due to inactivation of either the Ca^{2+} channels or the processes which link depolarization with exocytosis (Nordmann, 1976). In order to characterize

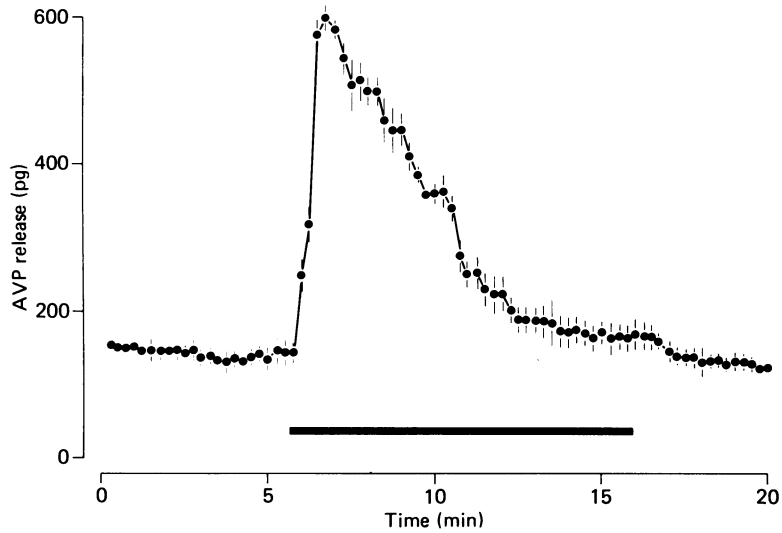

Fig. 4. Time course of AVP release from isolated nerve terminals depolarized with K^+. The neurosecretosomes were perfused with Na^+-free saline and stimulated with 100 mM-K^+ as indicated by the heavy bar. The fractions were of 15 s duration and the results are given as means ±S.E. of means ($n = 4$).

this phenomenon further, isolated nerve terminals were depolarized with K^+ for a prolonged period of time. In one group of experiments the neurosecretosomes were depolarized with 100 mM-K^+, whereas in the second group the terminals were exposed successively to increasing K^+ concentrations. The results are presented in Fig. 5. The results are expressed as rate constant of hormone release, since this takes into account the decrease in the hormonal content of the preparation during maintained secretion. Whereas 100 mM-K^+ gave rise to a large increase in AVP release which then decreased rapidly (Fig. 5*A*), the second paradigm gave successive increases of hormone secretion (Fig. 5*B*). This is of interest since it shows that upon successive increments of depolarization the process of hormone secretion from nerve endings can be reactivated. In these two types of experiments the K^+-evoked AVP release was $14{\cdot}1 \pm 1{\cdot}2$ ng ($n = 5$) and $20{\cdot}2 \pm 1{\cdot}0$ ng ($n = 4$), respectively. Thus, although the amount of hormone released at the onset of a depolarization with 100 mM-K^+ is larger than with a lower K^+ concentration, the total amount of peptide secreted was larger when the second paradigm was used.

Fig. 5 also shows that even after prolonged depolarization of the neurosecretosomes an increase of the cytoplasmic Ca^{2+} concentration, by permeabilization of the membrane with digitonin, reactivates the process of hormone secretion. The mechanism by which digitonin can induce an increase of hormone release in the presence of micromolar concentrations of Ca^{2+} will be discussed in the following paper (Cazalis *et al.* 1987).

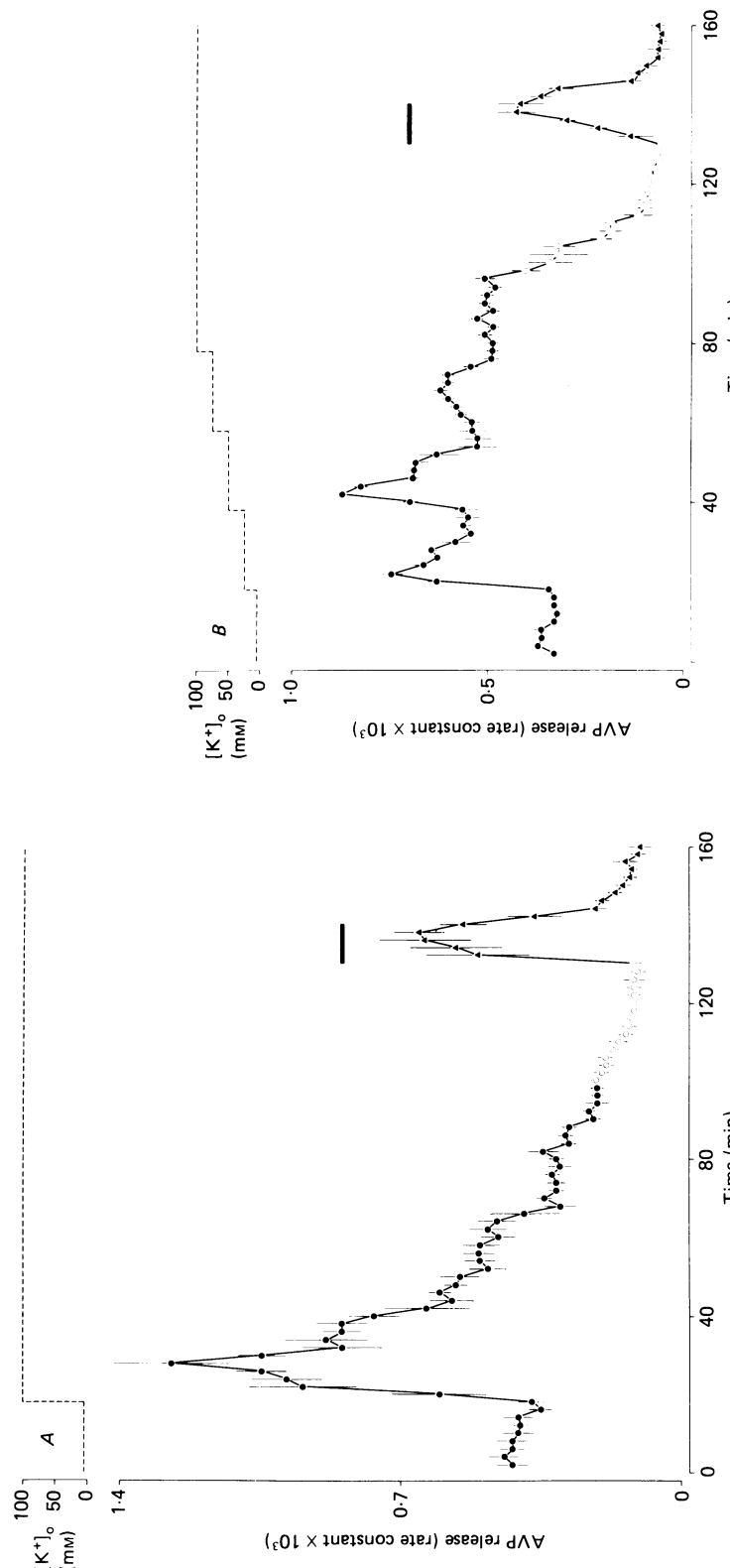

Fig. 5. The effect of prolonged K^+-induced depolarization on AVP release from isolated neurosecretory nerve endings. The K_o^+ was raised as indicated at the top of each panel. Note that in *A*, K_o^+ increased directly from 5 to 100 mM whereas in *B* depolarization was achieved with four subsequent changes of K_o^+. The preparation was perfused in the presence of 2·2 mM- (●) or 1·1 μM- (▲) Ca^{2+} with a Ca^{2+}-free medium (○). The heavy bar indicates the time during which the nerve endings were permeabilized with digitonin (for further details see Cazalis *et al.* 1987). The results are given as rate constant of AVP release and represent, for each panel, the means ± S.E. of means of four experiments. The rate constant is expressed as $\Delta H/\Delta t . H_t$ where ΔH represents the amount of hormone released in the time interval Δt and H_t the neurosecretosome hormone content at the mid-point of interval Δt.

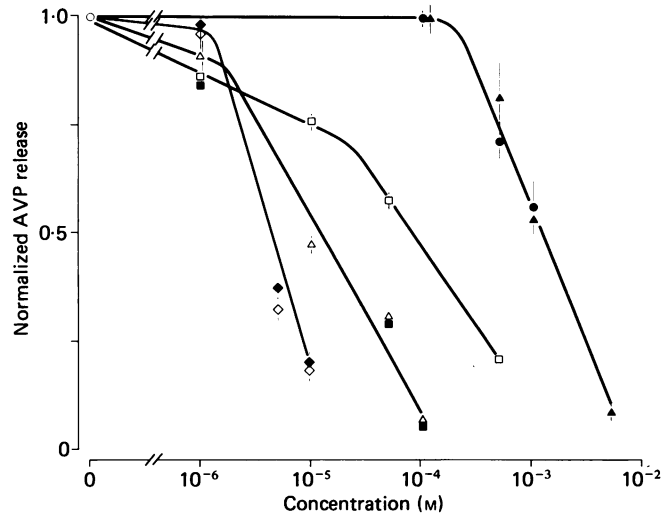

Fig. 6. The effect of Ca^{2+} antagonists on K^+-induced AVP release. The isolated nerve endings were perfused with 100 mM-K^+, Na^+-free Locke solution in the presence of different concentrations of Ca^{2+} antagonists. The hormone release induced by 10 min exposure to high K^+ was normalized by taking as unity the amount of AVP release in the absence of the Ca^{2+} antagonist. The Ca^{2+}-channel blocking agents were nicardipine (◇), nitrendipine (◆), D600 (△), Cd^{2+} (■), Gd^{3+} (□), Mn^{2+} (●) and Co^{2+} (▲). The lines were drawn by eye. The results represent the mean of three to eight experiments. Only standard errors of the mean larger than the symbols used are given.

The effect of Ca^{2+}-channel blocking agents

The effects of molecules or ions known to block Ca^{2+} channels has been studied on isolated neurosecretory nerve endings stimulated for 10 min with 100 mM-KCl in a Na^+-free medium. The perfusate was collected for a total period of 24 min after increasing K_o^+ and the evoked AVP release was calculated as described in the Methods. The Ca^{2+} antagonists were added 20 min before the onset of the stimulation and were present for the rest of the experiment. None of the Ca^{2+} antagonists used modified the basal hormone release. Fig. 6 illustrates the dose of dependence of K^+-induced AVP release from the nerve endings incubated with different Ca^{2+}-channel blocking agents. The order of effectiveness of the cations used to inhibit Ca^{2+}-dependent AVP release was $Cd^{2+} > Gd^{3+} > Co^{2+} = Mn^{2+}$, the typical concentrations necessary to effect half-maximal inhibition (IC_{50}) being about 10, 80, 1100 and 1100 μM respectively. The Ca^{2+} antagonist D600 (methoxyverapamil) had an IC_{50} of *ca.* 10 μM. Of interest is the observation that the dihydropyridine Ca^{2+} antagonists nitrendipine and nicardipine have a profound effect on K^+-induced hormone release. The concentration necessary to reduce the secretion by 50% was about 4 μM for both compounds.

Potentiation of AVP release by a Ca^{2+} antagonist

Although similar in structure to the Ca^{2+} antagonist nifedipine, Bay K 8644 has been shown recently to promote Ca^{2+} entry in different excitable tissues (Garcia,

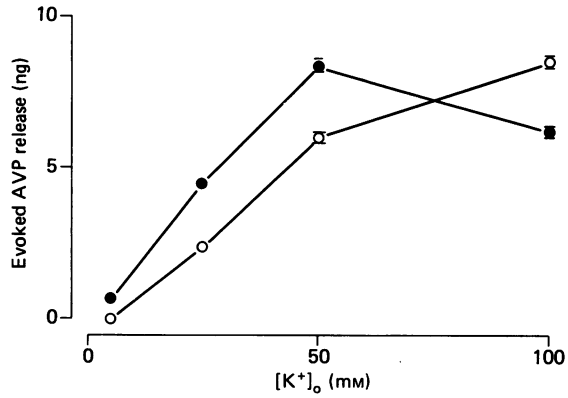

Fig. 7. The effect of the dihydropyridine Bay K 8644 on AVP release from isolated nerve endings. K_o^+ was increased in the presence of 5 μM (●) or absence (○) of the nifedipine derivative. The results are expressed as evoked AVP release and are given as the mean ± S.E. of mean of five to twelve experiments. Only the standard errors of the mean larger than the symbols used are given ($P < 0.001$ at any $[K^+]_o$).

Sala, Reig, Viniegra, Frias, Fonteriz & Gandia, 1984; Freedman, Dawson, Villereal & Miller, 1984; Greenberg, Carpenter & Cooper, 1985). Here we show that Bay K 8644 induced, during a period of 10 min, a small but significant increase in AVP release under basal conditions and that it potentiated the K^+-induced secretion. However this potentiation was observed with K^+ concentrations of 5, 25 and 50 mM but disappeared with depolarization induced with 100 mM-K^+ (Fig. 7). Under this depolarization condition Bay K 8644 had an inhibitory effect on AVP release. This inhibitory effect was totally abolished when Ca_o^{2+} was increased from 2·2 to 4·4 mM. These results suggest that Bay K 8644 modifies the state of the Ca^{2+} channel and that this modification is a function of the depolarized state of the membrane.

DISCUSSION

The present paper demonstrates that isolated neurohypophysial nerve endings can be used to study the mechanisms which couple the entry of external Ca^{2+} to the release of the neurosecretory product. It is of interest to know that neurosecretosomes were first isolated more than twenty years ago (La Bella & Sanwal, 1965) but that studies by many groups on such nerve terminals have been very disappointing (Baker, Vilhardt & Hope, 1975; Baker & Hope, 1976; J. J. Nordmann, unpublished). Although the neurosecretosomes have been shown to have a membrane potential which diminishes with increasing external K^+ concentration (Nordmann, Desmazes & Georgescault, 1982) previous studies failed to detect an enhanced release of hormones under such conditions. To our knowledge cold was the only stimulus which could induce the release of AVP from nerve terminals (Baker *et al.* 1975; Baker & Hope, 1976). Although we do not know exactly the differences between the present preparation and those used previously by us or by others, we can attempt to speculate on the nature of these differences. First, although they have a membrane potential,

neurosecretosomes prepared from bovine neural lobes might not secrete hormone upon depolarization because the release mechanism(s) has been somehow inactivated following the increased electrical activity of the magnocellular neurones during haemorrhage (see Poulain, Wakerley & Dyball, 1977) of the animals at the slaughterhouse. Dog neural lobes isolated after haemorrhage do not respond to K^+ depolarization (Sachs & Haller, 1968). Secondly, it seems that the presence of EGTA in the homogenization medium is important for allowing the nerve endings to respond subsequently to depolarization. We measured, using the fluorescent Ca^{2+} indicator Fura-2, that a medium without added Ca^{2+} and EGTA contains 2–10 μM-free Ca^{2+} (Brethes, Dayanithi, Letellier & Nordmann, 1987). This might be a high enough concentration to inactivate some internal mechanisms or to cause massive release during homogenization.

Hormone release under basal conditions

Vasopressin release from isolated neurosecretory nerve terminals was not affected by increasing Ca_o^{2+} from 2 μM to 2·2 mM. Under similar conditions the mean free Ca^{2+} concentration in the neurosecretosomes increases approximately from 110 to 350 nM (Brethes *et al.* 1987). Assuming that under these conditions the Ca^{2+} concentration is very similar at any point of the cytoplasm, these results suggest that the threshold for inducing an evoked increase in secretion is higher than 350 nM.

The reduction, under basal conditions, of the amount of hormone released following Na^+ substitution is somewhat puzzling. Under similar conditions basal release of AVP from the isolated neurohypophysis is potentiated (Douglas, 1974). However, the phenomena studied might not be exactly similar since, in contrast to the other study, the observed decrease in hormone release occurs rapidly after Na_o^+ removal (Fig. 1). A possible explanation would be that Na_i^+ will promote the entry of Ca^{2+} (Douglas & Poisner, 1964*a*; Lemos, Nordmann, Cooke & Stuenkel, 1986) which in turn will promote hormone release. This hypothesis is supported by the results of the experiments in which Na^+ was increased by means of the ionophore monensin. This molecule has been shown to increase catecholamine release from cultured adrenal medullary cells (Suchard, Lattanzio, Rubin & Pressman, 1982) and miniature end-plate potential frequency in the frog neuromuscular junction (Meiri, Erulkar, Lerman & Rahamimoff, 1981). The increase in AVP release observed in the presence of monensin could reflect an exchange of Na_i^+ for Ca_o^{2+}. The absence of effect of ouabain on AVP release from neurosecretosomes could be the result of a lower Na_i^+ concentration, and hence a lower rate of Na^+–Ca^{2+} exchange. Clearly, the precise role of Na_i^+ on neurohormone release remains to be determined.

The maintained AVP release during prolonged exposure to the Na^+ ionophore is of interest. Whereas maintained K^+ (Muller, Thorn & Torp-Pedersen, 1975; Nordmann, 1975, 1976) or veratridine-induced (Dyball & Nordmann, 1977) depolarization give rise to an increase in hormone release which wanes with time, monensin produces a small but maintained rate of secretion. In contrast to prolonged K^+ depolarization which releases a maximum of about 16% of the total hormone content, incubation with monensin for a period of 4 h gives rise to the release of about 36% of the stored hormone. Similarly, in cultured adrenal medullary cells the maximum response to carbachol stimulation occurs within 6–10 min of exposure to

the receptor agonist and 25–35% of the total catecholamine are secreted. In contrast, the response to monensin is linear for 6 h, by which time the cells have released 75–90% of their total catecholamine content (Suchard *et al.* 1982).

Hormone release under depolarized conditions

The isolated nerve endings respond to increased K_o^+, as observed with the neurohypophysis. One of the main advantages in using the neurosecretosomes is the rate at which an increase (or a decrease) of hormone release can be observed. For example, with the perfusion system used in this study it took only *ca.* 5 min for hormone release, after reaching its maximum, to return to its basal level. This is much shorter than the time for the neural lobe in which the slower falling phase is likely to be due to hormone accumulation in the extracellular space (Muller *et al.* 1975). Furthermore, a relatively high rate of perfusion allowed us to measure, after a first increase, the rapid decay of secretion during K^+-induced stimulation. This is of importance for it shows that, during depolarization, it takes about 60 s to reach a maximal hormone release and the release than decreases rapidly ($t_{\frac{1}{2}} = 2{\cdot}5$ min), although the nerve endings remain depolarized (Nordmann *et al.* 1982). This value is much smaller than that measured previously with the intact neurohypophysis (Nordmann, 1976).

The evoked hormone release induced by veratridine is similar to that observed with the nerve endings *in situ*. The results confirm the presence of Na^+-channels in the membrane of the neurosecretosomes (Nordmann & Dyball, 1978; Dellmann, Boudier, Couraud, Cau & Boudier, 1983). The pathway by which hormone secretion is triggered involves the entry of Na^+, depolarization of the nerve endings, and entry of Ca^{2+} through the Ca^{2+} channel.

Ca^{2+} channels in the isolated nerve endings

The presence of Ca^{2+} channels has been demonstrated in a large variety of excitable tissues (for a review see Hagiwara & Byerly, 1981). It seems that most of the Ca^{2+} ions involved in the mechanism of secretion penetrate through the 'late' Ca^{2+} channel first described in the squid axon (Baker *et al.* 1973). As observed in other tissues, Co^{2+} and Mn^{2+} block $^{45}Ca^{2+}$ uptake and hormone release in the isolated neurohypophysis (Dreifuss *et al.* 1973; Nordmann, 1976). Here we present further evidence for the blocking effect of Ca^{2+} entry and hormone release by divalent and trivalent cations. We found that in the neurosecretory nerve endings Cd^{2+} ($IC_{50} = 10\ \mu M$) and Gd^{3+} ($IC_{50} = 80\ \mu M$) have a more potent inhibitory effect on secretion than Mn^{2+} or Co^{2+}. Similarly Gd^{3+} has been shown to be a potent inhibitor of $^{45}Ca^{2+}$ uptake and catecholamine release from cultured chromaffin cells (Bourne & Trifaro, 1982). It has also been found recently that Gd^{3+} inhibits the evoked-AVP release from the rat neurohypophysis (Muscholl, Racké & Traut, 1985). We also observed that D600 (methoxyverapamil) has an apparent inhibitory constant ($IC_{50} = 10\ \mu M$) very similar to that calculated from experiments performed on the isolated neural lobe (Nordmann, 1983). The effect of D600 on AVP release can be associated with its blocking action on Ca^{2+} entry during depolarization of the neurosecretosomes as measured with Fura-2 (Brethes *et al.* 1987). The exact nature of the action of D600 is still debatable (Terakawa, 1981; Clay & Shrier, 1984; Hescheler, Pelzer, Trube & Trautwein, 1982). Non-specific effects have been reported with high concentrations of

D600 (Galper & Cotterall, 1979; McGee & Schneider, 1979). As yet we do not know the precise site of interaction of D600 with the mechanism of stimulus–secretion. From the above considerations, it would seem plausible that at relatively low concentration ($< 100\ \mu$M) D600 mainly blocks the depolarization-induced Ca^{2+} uptake through the Ca^{2+} channel. However, more specific Ca^{2+} blocking agents such as the dihydropyridines (Fig. 6) might be more suitable for blocking Ca^{2+} channels and hence depolarization-induced neurohormone release.

Dihydropyridines have recently been shown to modify Ca^{2+} channel activity in excitable cells (Reuter, Porzig, Kokubun & Prod'hom, 1985). Some dihydropyridines act as antagonists whereas other derivatives appear to potentiate the Ca^{2+} current through voltage-dependent Ca^{2+} channels (Hess, Lansman & Tsien, 1984). Most of the dihydropyridines block depolarization-induced release from secretory cells, but the nifedipine derivative Bay K 8644 has been shown to promote the K^+-evoked catecholamine release from cultured adrenal medullary cells (Garcia *et al.* 1984; Shalaby, Kongsamut, Freedman & Miller, 1984). Furthermore, in a pituitary cell line Bay K 8644 by itself can stimulate secretion of prolactin (Enyeart & Hinkle, 1984). Our results show that the dual mode of action of the dihydropyridines can also be observed in isolated neurosecretory nerve endings. Nitrendipine and nicardipine were extremely potent in reducing the evoked AVP release during K^+ stimulation. Their apparent inhibitory constant was about 4 μM, which is likely to render them extremely useful for further studies on neurosecretion. Interestingly, nifedipine itself had no effect on hormone release. This result is similar to those of Nachsen & Blaustein (1979) and Daniell, Barr & Leslie (1983) who reported the lack of effect of this molecule on $^{45}Ca^{2+}$ uptake in brain synaptosomes. Although care was taken to prevent possible light-induced degradation of nifedipine, the lack of effect of this molecule on AVP release should be interpreted with caution as the experiments with the nifedipine derivative Bay K 8644 suggest that the nerve endings have a receptor for this class of molecules.

As observed in experiments with the neural lobe, prolonged K^+-induced depolarization of the neurosecretosomes gives rise to an increase in hormone release which decreases with time. In the neural lobe, the decrease in hormone release occurs at a higher rate than that of depletion of the storage organelles (Nordmann, 1975, 1976). Similarly, in the present study we found that during prolonged K^+-induced depolarization reactivation of secretion can be observed following permeabilization of the neurosecretosomes in the presence of micromolar concentration of Ca^{2+}. This reactivation can be explained by the increase of Ca_i^{2+} which follows the permeabilization with digitonin. Similar reactivations have been observed by introducing, during prolonged depolarization of the neurohypophysis, Ba^{2+}, the ionophore X537A (Nordmann, 1976), veratridine (Dyball & Nordmann, 1977) or by reducing the Na_o^+-concentration (Muller *et al.* 1975; Nordmann, 1976). Of interest is the observation that the pattern of hormone release induced with a 'stepwise' increase of K_o^+ (Fig. 5*B*) is different from that obtained after abruptly raising to 100 mM the K_o^+ concentration (Fig. 5*A*). The decrease in secretion observed after prolonged depolarization with 25 mM-K^+ is followed by an increase of hormone release when the nerve endings are subsequently perfused with 50 mM-K^+. Similar increases, although smaller, are observed with further depolarization. Moreover, the 'stepwise'

paradigm gives rise to more hormone release than that observed after depolarization, induced with 100 mM-K^+, at the onset of the stimulus.

A tentative explanation for these results is as follows. After maximal depolarization of the nerve endings the cytoplasmic ionized Ca^{2+} concentration increases and this triggers a large amount of hormone release. Although the depolarization is maintained, the Ca^{2+} channels do inactivate, and, due to the buffering capacity of the nerve endings, Ca_i^{2+} decreases and secretion is not maintained. The 'stepwise' paradigm promotes, although to a smaller extent, an increase of Ca_i^{2+}, and at each depolarizing step a new subpopulation of channels opens and give rise to a further increase in hormone secretion. However, as will be discussed in the following paper (Cazalis *et al.* 1987), another alternative which is not mutually exclusive is that the important parameter for inducing hormone secretion is not only the absolute concentration of Ca_i^{2+}, but also the rate at which the Ca^{2+} concentration, or another parameter at active sites, varies. This hypothesis has been discussed by Knight & Baker (1982) who have analysed catecholamine release from permeabilized chromaffin cells. In the experiments described in the present paper the larger amount of hormone secreted with the 'stepwise' paradigm could thus be explained by the multiple changes of Ca_i^{2+} induced by this type of experiment.

The recent finding that the neurosecretosomes isolated from the rat neurohypophysis can be studied using the patch-clamp technique (Lemos & Nordmann, 1986) and the present study suggest that the isolated neurosecretory nerve endings from the neural lobe might be of great interest for studying the coupling between electrical activity and the events leading to the exocytosis of neurohormones.

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