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SODIUM-EVOKED, CALCIUM-INDEPENDENT VASOPRESSIN RELEASE FROM RAT ISOLATED NEUROHYPOPHYSIAL NERVE ENDINGS

BY EDWARD L. STUENKEL AND JEAN J. NORDMANN

From the Department of Physiology, University of Michigan, Ann Arbor, MI 48109-0622, USA and Centre de Neurochimie, 5 Rue Blaise Pascal, 67084 Strasbourg Cedex, France

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SUMMARY

1. The effects of Na^+ on vasopressin release and on redistribution of Ca^{2+}, Na^+ and H^+ in isolated rat neurohypophysial nerve endings have been studied.

2. Substituting Na^+ for a non-permeant cation produced a pronounced and sustained release of vasopressin. This increase occurred in the absence of external Ca^{2+} and in nerve endings loaded with the Ca^{2+} chelator dimethyl-BAPTA (1,2-bis-(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid).

3. The effect of Na^+ was independent of a rise in intracellular Ca^{2+} as judged by the measurement of $[Ca^{2+}]_i$ using the indicator fura-2 and $^{45}Ca^{2+}$ efflux studies. Although Na^+ could release Ca^{2+} from internal reservoirs the small elevation in $[Ca^{2+}]_i$ induced by Na^+ could not explain the large and sustained increase in vasopressin secretion.

4. The channel blockers TTX (tetrodotoxin), D888 (desmethoxyverapamil), N144 (5-nitro-2-(phenylpropylamino)-benzoic acid) or SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid) could not prevent the Na^+-dependent increase in vasopressin release. Similarly this increase was not affected by metabolic inhibitors (Ruthenium Red and KCN) nor by CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), an uncoupler of oxidative phosphorylation.

5. Selectivity among monovalent cations to promote secretion was found with the largest effect on the secretory response being produced by Na^+. Similarly Cl^- was found to be the most potent anion studied for inducing, in the presence of Na^+, an increase in neurohormone release.

6. Measuring $[Na^+]_i$ by means of the Na^+ indicator SBFI showed that the extent of the secretory response was correlated with the intraterminal Na^+ concentration.

7. The Na^+-induced, Ca^{2+}-independent release of vasopressin occurred by exocytosis as judged (i) by the linear relationship between the amount of vasopressin secreted and that of the co-localized neurophysin and (ii) by the demonstration that the extracellular marker horseradish peroxidase was only found in endocytotic vacuoles and not in the cytoplasm of the stimulated nerve endings.

8. The Na^+-dependent secretory response found on addition of extracellular Na^+ was not the result of the change in internal pH as measured with the indicator BCECF and as mimicked by addition of propionic acid.

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9. Addition of Na^+ to digitonin- or streptolysin-O-permeabilized nerve endings in the presence or absence of Ca^{2+} also gave rise to an increase in vasopressin secretion.

10. It is concluded that an increase in internal Na^+ *per se* can promote, in the absence of a rise in intracellular Ca^{2+}, an increase in neuropeptide secretion.

INTRODUCTION

It has become universally accepted that regulated secretion from neurons is triggered by alterations of intracellular Ca^{2+} concentration. In the preceding paper (Stuenkel & Nordmann, 1993) we have shown that in isolated nerve endings from the rat neurohypophysis, not only is the *increase* in $[Ca^{2+}]_i$ important for the rate and the extent of secretion but that periodic *changes* in the $[Ca^{2+}]_i$ are also an important parameter in regulating the secretory mechanism. Furthermore, we have found that in the neurohypophysial nerve endings a maintained high $[Ca^{2+}]_i$ was not sufficient to sustain a high rate of secretion. A large number of other factors have been reported to exert a regulatory influence on neurotransmitter and neurohormone secretion. In many cases the level and extent of secretion has been shown to be variously modulated by the electrical activity of neurones (Magleby, 1973; Magleby & Zengel, 1982; Zucker, 1989). A change in intracellular Na^+ has been implicated in the mechanism of facilitation and post-tetanic potentiation of transmitter release at neuromuscular junctions of different species (Sherman & Atwood, 1971; Atwood, Swenarchuck & Gruenwald, 1975; Swenarchuck & Atwood, 1975; Ortiz, 1980; Nussinovitch & Rahamimoff, 1988). The resulting effects of Na^+ to enhance neurotransmitter release have been further substantiated through sodium loading of nerve endings by liposome fusion at the neuromuscular junction of the frog (Rahamimoff, Meiri, Erulkar & Barenholz, 1978) or by direct injection of Na^+ in the squid giant synapse (Charlton & Atwood, 1977). A role of Na^+ has also been shown in the mechanism leading to the release of various amino acid neurotransmitters (for review see Bernath, 1992; Adam-Vizi, 1992) as well as for catecholamines (Suchard, Lattanzio, Rubin & Pressman, 1982). Aside from the involvement of specific Na^+-coupled transport mechanisms for some of the neurotransmitters (Woodward, Chandler & Leslie, 1988; Okada, Mine & Fujiwara, 1990) the enhancement of neurotransmitter release by Na^+ has been related to the ability of Na^+ to change the intracellular $[Ca^{2+}]_i$ in nerve endings (Satoh & Nakazato, 1991) or to alter the affinity of Ca^{2+} for the exocytotic Ca^{2+} receptor (Ortiz, 1980).

In the present study we have re-examined the effect of Na^+ on the exocytotic release of vasopressin from isolated nerve endings. We report here a possible direct action of Na^+ on the machinery leading to exocytosis of neurohormone. Preliminary accounts have already been published (Nordmann & Stuenkel, 1991; Stuenkel & Nordmann, 1991).

METHODS

Preparation and perfusion of isolated nerve endings

Isolated rat neurohypophysial nerve endings were prepared and perfused as described previously (Cazalis, Dayanithi & Nordmann, 1987*a*). Briefly the nerve endings were loaded onto filters (0·45 μm Acrodisk LCPVDF, Gelman, Ann Arbor, MI, USA) and perfused with normal physiological saline at a slow rate (25 μl min^{-1}). After 30 min the rate of perfusion was increased to 50 μl min^{-1}

and the nerve endings were perfused either in 40 mM Na^+ saline or in NM medium or in cytoplasmic-like medium (see below). Collection of fractions was started 90 min after loading of the nerve endings onto the filters. AVP content of the fractions was determined by radioimmunoassay as described (Cazalis, Dayanithi & Nordmann, 1985).

Permeabilization of the nerve endings

Permeabilization of the nerve endings with digitonin or streptolysin-O was performed as described in Cazalis, Dayanithi & Nordmann (1987*b*; see also Stuenkel & Nordmann, 1993).

Electron microscopy

After incubation in normal saline the isolated nerve endings were centrifuged at $2400 \times g$ for 4 min and resuspended in NM medium (see below) for a period of 45 min. Horseradish peroxidase (HRP, 5 mg ml^{-1}) was added and the nerve endings were centrifuged before being resuspended for 10 min either in NM (control) or in Na^+ medium both containing HRP. The nerve endings were again centrifuged (15 min) and the pellet fixed in 2·5% gluteraldehyde-containing sodium cacodylate (120 mM; pH 7·2) buffer. Diamino-benzidine tetrahydrochloride was used as a substrate for reaction with horseradish peroxidase as described by Graham & Karnovsky (1966). The nerve endings were post-fixed in 1% OsO_4 for approximately 45 min, dehydrated in a series of ethanol and embedded in Spur resin.

Radiotracer studies

$^{45}Ca^{2+}$ and $^{86}Rb^+$ efflux: the nerve endings were preincubated for 60 min in normal physiological saline containing 10 μCi or 1 μCi ml^{-1} of $^{45}Ca^{2+}$ or $^{86}Rb^+$ respectively (Amersham, France). They were then loaded onto filters and perfused with NM at a flow rate of 50 μl min^{-1}. Fractions were collected in 2 min intervals. Efflux was expressed as a rate constant k (min^{-1}) = $\Delta X/(tX_t)$, where ΔX represents counts $^{45}Ca^{2+}$ or $^{86}Rb^+$ released in the mean time interval Δt and X_t the tissue content at the midpoint of interval Δt. The tissue content of the radioactive tracer at the end of the experiment was determined by perfusing 2 ml of Triton X-100 (0·5%) through the filter and by counting the radioactivity in the eluant.

Fluorescence measurements

Measurement of intracellular [Ca^{2+}], [Na^+] or [H^+] was performed on individual isolated nerve endings using the ion-sensitive, fluorescent probes fura-2, SBFI and BCECF (2′,7′-bis(carboxyethyl)-5-carboxyfluorescein), respectively. Loading of the probes into the isolated nerve endings was accomplished by incubation with the acetoxymethyl derivatives, fura-2 AM (1·5 μM, 20 min), SBFI AM (5 μM with 0·05% pluoronic acid, 40 min) and BCECF AM (5 μM, 30 min) in normal physiological saline. Following loading, the nerve endings were centrifuged at $2400 \times g$ for 4 min to remove extracellular indicator, resuspended in normal physiological saline and allowed to sit for 20 min to allow for complete cleavage of the ester group. Spectroscopic monitoring of fluorescence on individual nerve endings for each indicator was performed using a photomultiplier-based Spex system (Spex Industries, Edison, NJ, USA) coupled to a Nikon microscope as described previously (Stuenkel & Ernst, 1990; Stuenkel, 1990; Stuenkel & Nordmann, 1993). Excitation wavelengths of 340 ± 10 and 380 ± 10 nm were used for fura-2 and SBFI while 440 ± 10 and 490 ± 10 nm were used for BCECF. Emission was monitored at 510 ± 10 nm (or at 500 ± 20 nm) and at 520 ± 10 nm for fura-2 and SBFI and for BCECF, respectively. Calibration of the fluorescent ratios into the appropriate ionic concentration were performed either with an external standards calibration (fura-2) or by an internal standards approach (SBFI, BCECF). Conversion of the fura-2 340/380 nm ratio to $[Ca^{2+}]_i$ was performed using the equation given by Grynkiewicz, Poenie & Tsien (1985). For SBFI the Na^+ ionophore monensin (10 μM) was used in conjunction with perfusion of media containing given $[Na^+]$ to set $[Na^+]_o$ equal to $[Na^+]_i$. It should be noted that for $Na^+ \leqslant 40$ mM the correlation of the fluorescence ratio to concentration was high (regression coefficient of $\geqslant 0{\cdot}92$) while above 40 mM Na^+ the calibration was less accurate (regression coefficient $\geqslant 0{\cdot}80$). Calibration of BCECF fluorescence was performed using the K^+–H^+ exchanger nigericin to set cytoplasmic pH equal to the pH of the external medium as described previously (Stuenkel, Machen & Williams, 1989).

RESULTS

Effects of Na^+ on the release of AVP

The first series of experiments examined the effect of changes of extracellular Na^+ on the release of AVP. In order to isolate the effect of Na^+ ions the nerve endings were perfused for a period of 45 min in a medium lacking permeant cations and containing (mM): *N*-methyl-D-glucamine (NMG^+)-Cl, 140; Tris-Hepes, 10, pH 7·2; glucose, 10; EGTA, 0·1; and bovine serum albumin (BSA), 0·01% (see Methods). This medium was named 'nothing medium', based on the absence of highly permeant cations, and hereafter will be referred to as NM. Following the period in NM equimolar substitution of Na^+ for NMG^+ gave rise to a robust secretory response exhibiting an average peak increase of $7·0 \pm 0·8$ ($n = 28$)-fold compared with basal release. A representative example is illustrated in Fig. 1*A*. After returning the nerve endings to NM a second response was elicitated by re-exposure of the neurosecretosomes to Na^+-containing medium. Compared with the brief Ca^{2+}-dependent transient secretory response described in the preceding paper (Stuenkel & Nordmann, 1993) a major finding was that during prolonged perfusion with Na^+-containing solutions the secretory response remained substantially elevated, declining only slowly ($t_{\frac{1}{2}} > 1$ h, $n = 6$) (Fig. 1*A*). Na^+-containing solutions could repetitively induce AVP release following NM perfusion as illustrated in Fig. 1*B*. In this series of experiments the nerve endings were challenged with eleven pulses of Na^+-containing medium of 20 min periods each (Fig. 1*B*). In the experiment shown Na^+ induced a total evoked release of 6·2 ng of AVP which represented 5·8% of the tissue content at the beginning of the experiment. The amplitudes of the Na^+-induced secretory responses were very similar, suggesting that release did not result from lysis of the nerve endings (see also below). Furthermore, in a parallel series of experiments the amplitude of the secretory response induced by K^+ stimulation at the end of an 11 h period of incubation was very similar to that induced by depolarization of the nerve endings after 1 h (not illustrated). This confirms that even after a prolonged period of perfusion the nerve endings are still competent to respond to a depolarizing secretory stimulus.

In order to compare the amplitude of the K^+-induced, Ca^{2+}-dependent, secretory response with that observed after increasing the extracellular Na^+ concentration, the nerve endings were first depolarized with 50 mM K^+ during a period of 8 min. They were then incubated in NM for 80 min and release of AVP was triggered by substitution of Na^+ for NMG^+. Figure 1*C* illustrates the results and shows that Na^+ ions can induce a secretory response of peak amplitude similar to the approximately 4-fold increase (see also Cazalis *et al.* 1987*a*) generated by the Ca^{2+}-dependent K^+-induced response.

Effects of Na^+ and Ca^{2+} on the release of AVP

In a series of experiments we tested if external Ca^{2+} and Na^+ ions interact to potentiate release of AVP after perfusion of the nerve endings in NM. Figure 2*A* illustrates an experiment in which the nerve endings were first challenged with a medium containing (mM): NaCl, 140; $CaCl_2$, 2·2; Tris-Hepes, 10, pH 7·2; EGTA, 0·1; glucose, 10; BSA, 0·01%. After return to NM they were challenged a second time with the same medium but lacking $CaCl_2$. The amplitude and the time course of AVP

release were very similar in both cases demonstrating that the Na^+-induced response is not potentiated by Ca^{2+} ions. In another series of experiments the nerve endings were first challenged with 2·2 mM Ca^{2+}. After return to Ca^{2+}-free NM they were perfused with Ca^{2+}-free, Na^+-containing saline. The results are illustrated in Fig. 2*B*

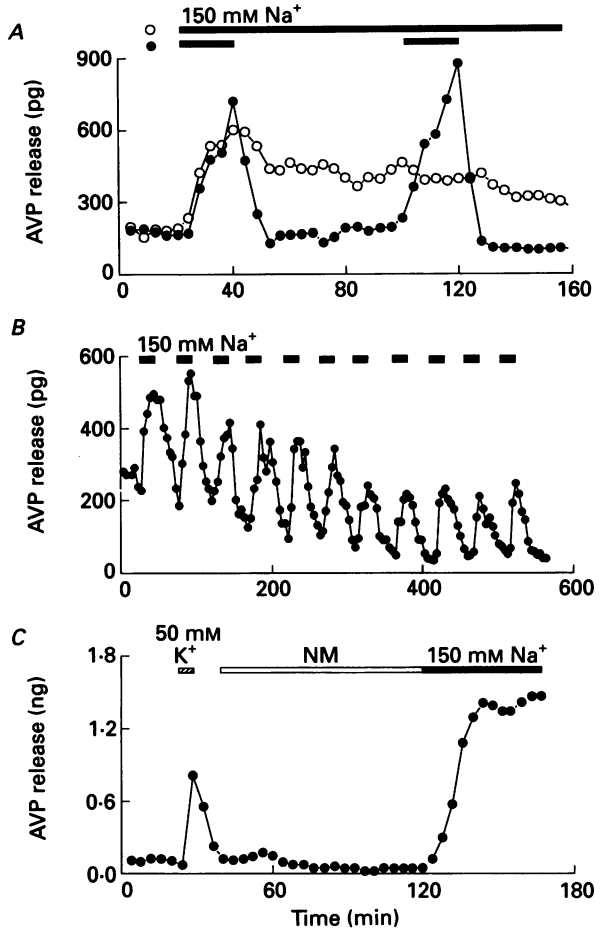

Fig. 1. Effects of Na^+ on the release of AVP from isolated nerve endings. Nerve terminals were perfused with NM consisting of (mM): *N*-methyl-D-glucamine (NMG)-Cl, 140; Tris-Hepes, 10, pH 7·2; glucose, 10; EGTA, 0·1; BSA, 0·01%. *A*, effects on AVP release of substituting Na^+ for NMG^+ during two successive (●) and a prolonged (○) period of time. *B*, effects on AVP release of substituting Na^+ for NMG^+ during eleven periods of 20 min each. Similar observations were obtained on seven separate preparations. *C*, the nerve endings were first depolarized with 50 mM K^+-containing saline. They were then perfused with NM followed by Na^+ medium. Solution changes in this and all subsequent figures are as indicated by bars above graph.

and show that when given *before* the Na^+ challenge Ca^{2+} ions can promote secretion but the secretory response is not maintained. In contrast, as already mentioned above, perfusion of the same nerve endings with Na^+-containing, Ca^{2+}-free medium triggered a release response which declined with a much slower rate than that elicited

by calcium. Comparison of the Ca^{2+}- with Na^+-dependent secretory responses shows that the Ca^{2+}-dependent release is $30 \pm 5\%$ ($n = 3$) of the Na^+-dependent response. Furthermore, we systematically observed that when the nerve endings were first stimulated with Na^+-containing, Ca^{2+}-free medium ($4{\cdot}5 \pm 0{\cdot}4$-fold increase, $n = 3$), a

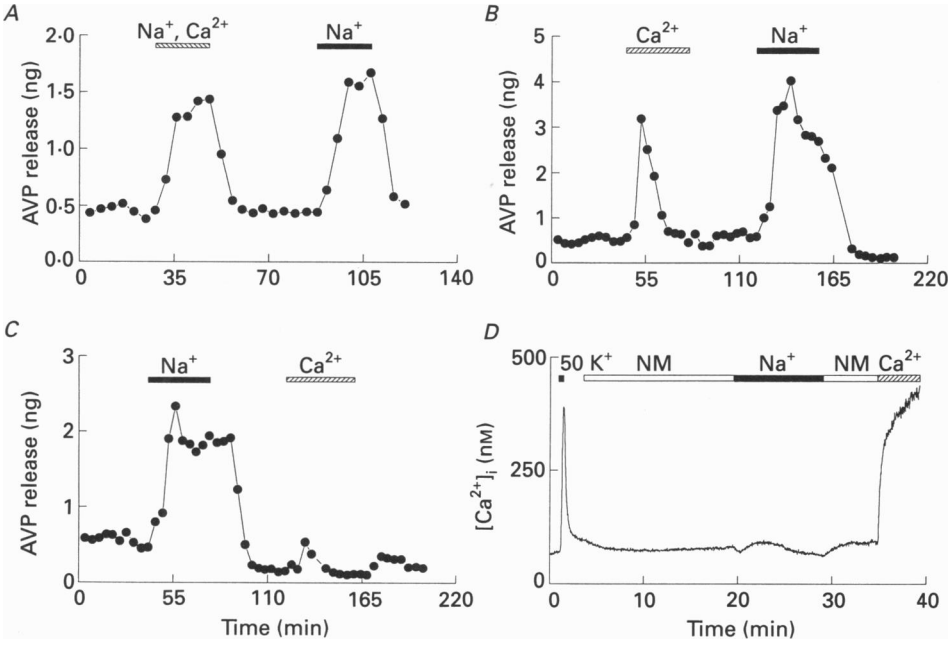

Fig. 2. Effects of Na^+ and Ca^{2+} on $[Ca^{2+}]_i$ and the release of AVP from isolated nerve endings. *A*, following perfusion in NM the nerve terminals were perfused with modified Na^+ medium in which EGTA was omitted and Ca^{2+} was added at a concentration of 2·2 mM. After return in NM the nerve endings were perfused with Na^+ medium. *B*, AVP release from nerve terminals challenged first with NM containing 2·2 mM Ca^{2+} and then with Ca^{2+}-free, Na^+-containing medium. *C*, AVP release from nerve terminals challenged first with Na^+ medium and then with NM containing 2·2 mM Ca^{2+} and no EGTA. *D*, $[Ca^{2+}]_i$ from a single nerve ending first depolarized with 50 mM K^+ as indicated and perfused then with NM. The perfusion was then switched to Na^+ medium. After return to NM, Ca^{2+} was added at a concentration of 2·2 mM. Results similar to those shown were observed in three (*A* and *B*), two (*C*) and twenty-eight (*D*) experiments.

second challenge in the same endings with a medium lacking Na^+ but containing Ca^{2+} gave rise to a small increase ($2{\cdot}3 \pm 0{\cdot}2$-fold increase) in secretion. A representative example is shown in Fig. 2*C* in which Na^+ induced a large and sustained release of AVP whereas Ca^{2+} triggered a small and transient secretory response.

The mechanism by which Na^+ exerts its secretory effect could be by stimulating Ca^{2+} release from intracellular stores resulting from Na^+ influx (Swenarchuck & Atwood, 1975; Alnaes & Rahamimoff, 1975; Rahamimoff, Lev-Tov & Meiri, 1980). To examine this possibility we monitored the intracellular calcium concentration ($[Ca^{2+}]_i$) in single isolated nerve endings using the Ca^{2+}-sensitive indicator fura-2. The results are illustrated in Fig. 2*D*. NM alone did not significantly reduce the $[Ca^{2+}]_i$. Substitution of Na^+ medium for NM induced a slight increase in $[Ca^{2+}]_i$ (50 ± 11 nM,

$n = 26$) that was substantially less than that evoked by a depolarizing K^+ stimulus in the same nerve endings. However, 2·2 mM Ca^{2+}-containing NM induced a large and sustained increase in $[Ca^{2+}]_i$. Note that with a similar protocol calcium produced only a modest increase in AVP release (Fig. 2*C*).

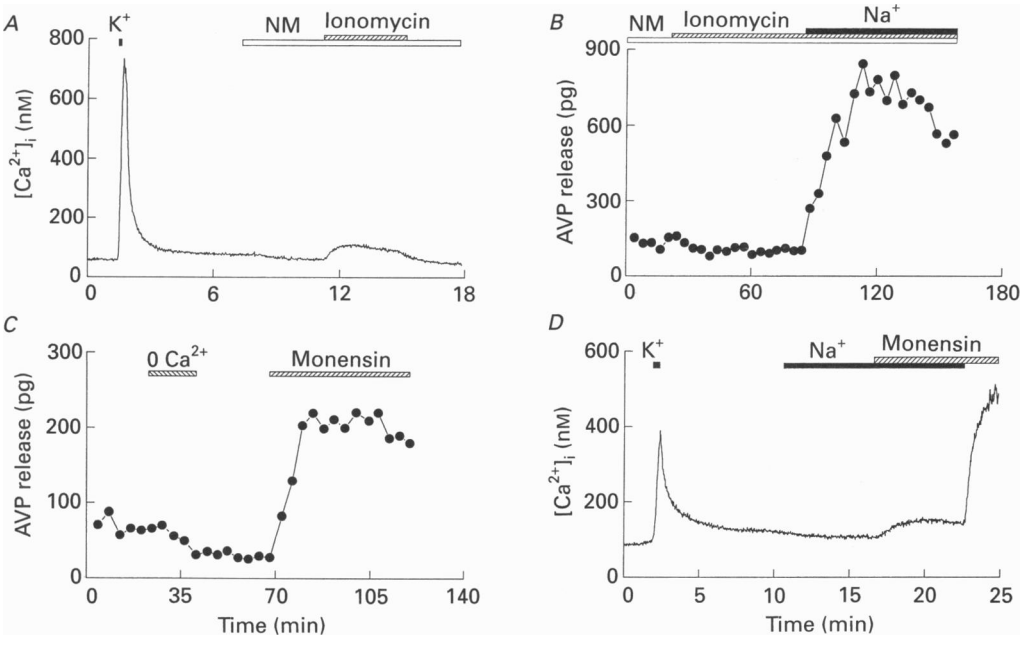

Fig. 3. Effects of Na^+ and Ca^{2+} ionophores on $[Ca^{2+}]_i$ and the release of AVP. *A*, $[Ca^{2+}]_i$ in a nerve ending first depolarized with 50 mM K^+ (40 mM Na^+ Locke solution). Perfusion was then in NM to which ionomycin was added (5 μM) as indicated. *B*, AVP release from nerve terminals perfused with NM to which 5 μM ionomycin was added as indicated. This was followed by perfusion with ionomycin containing 150 mM Na^+ medium. *C*, AVP release from nerve terminals perfused with Locke solution. Ca^{2+} and monensin (10 μM) were respectively omitted and added as indicated. *D*, $[Ca^{2+}]_i$ in a single nerve ending first depolarized with 50 mM K^+ and then perfused with Locke solution. Perfusion was then with Na^+ medium to which monensin (10 μM) was added as indicated. The nerve endings were returned to Locke saline at the end of the experiment.

Intracellular Ca^{2+} stores and AVP release

The experiments illustrated in Fig. 3 show that, under basal conditions, the neurohypophysial nerve endings are unlikely to contain large Ca^{2+} reservoirs. This is evidenced by the failure of ionomycin, a Ca^{2+} ionophore, to elicit a substantial increase in $[Ca^{2+}]_i$ when applied to nerve endings in the absence of external Ca^{2+} (Fig. 3*A*). Whereas K^+-induced-depolarization triggered a large rise in $[Ca^{2+}]_i$, addition of ionomycin in the absence of Ca^{2+} resulted in only a small increase in $[Ca^{2+}]_i$. Furthermore, addition of ionomycin to NM did not increase AVP release ($n = 10$). Note that following depletion of the intracellular Ca^{2+} stores by ionomycin, substitution of Na^+ for NMG^+ still is capable of triggering a large and sustained increase in secretion (Fig. 3*B*).

To investigate the possibility that there exist intracellular Ca^{2+} stores sensitive to alterations of the $[Na^+]_i$ we examined the effects of the Na^+ ionophore monensin on $[Ca^{2+}]_i$. Whereas removal and replacement of Ca^{2+} alone had no detectable effect on the amount of AVP release in the presence of Na^+, addition of the Na^+ ionophore

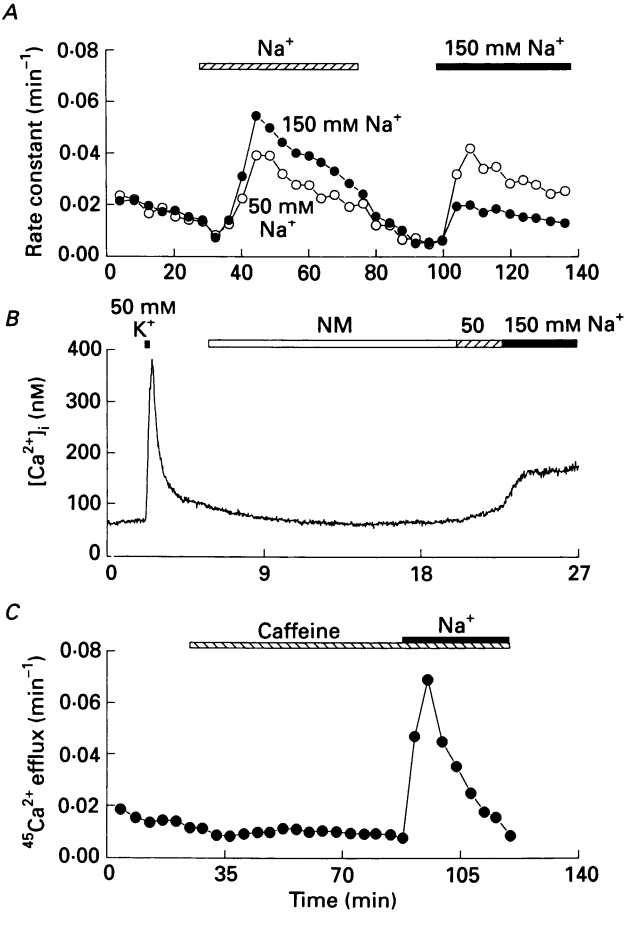

Fig. 4. Effects of Na^+ on the release of Ca^{2+} from internal reservoirs. *A*, $^{45}Ca^{2+}$ efflux from preloaded nerve endings perfused with NM and challenged first with 50 mM (○) or 150 mM Na^+ (●). After return to NM the nerve endings were perfused with 150 mM Na^+. The results are expressed as rate constants (min^{-1}). *B*, $[Ca^{2+}]_i$ as measured with fura-2. A single isolated nerve ending was first depolarized for 30 s with 50 mM K^+ and perfused with NM. 50 mM and 150 mM Na^+ were successively substituted for the equimolar concentration of NMG^+. *C*, $^{45}Ca^{2+}$ efflux from preloaded nerve endings perfused with NM to which caffeine (10 mM) was added. Na^+ was substituted for NMG^+ as indicated.

monensin to Na^+ medium induced a large and sustained increase (7·2-fold at peak increase, $n = 2$) in the secretory response (Fig. 3*C*). However, Fig. 3*D* shows that addition of monensin to Na^+ medium resulted in only a small rise in $[Ca^{2+}]_i$. Note that subsequent addition of calcium (2·2 mM) to the Na^+ medium produced a large increase in $[Ca^{2+}]_i$. The results demonstrate that the rise in $[Ca^{2+}]_i$ induced by

releasing Ca^{2+} from intracellular stores is of small amplitude. Additionally, the intracellular Ca^{2+} stores probably play little role in the Na^+-evoked secretory response as Na^+ induces marked secretion following depletion of these Ca^{2+} stores. The release of Ca^{2+} in the presence of monensin, however, was suggestive of a small intracellular Ca^{2+} pool that is sensitive to alterations in intracellular $[Na^{2+}]_i$.

The Na^+-dependent release of Ca^{2+} from internal reservoirs was further analysed by measuring $^{45}Ca^{2+}$ efflux from preloaded nerve endings. The isolated nerve endings were perfused with NM and challenged twice with Na^+. Figure 4*A* shows that Na^+ induced a dose-dependent efflux of $^{45}Ca^{2+}$. When in four experiments the nerve endings were initially challenged with 150 mM Na^+, a second stimulation with 150 mM Na^+ resulted in a smaller increase in efflux. Note, however, that under similar conditions there was no significant difference(s) between the Na^+-evoked secretory responses (see Fig. 1*A*). Parallel experiments performed to analyse changes in $[Ca^{2+}]_i$ using fura-2 show that substitution of 50 mM Na^+ for NMG^+ induced a small increase, compared to that observed during K^+-induced depolarization (Fig. 4*B*). Total replacement of NMG^+ by Na^+ produced a further small rise in $[Ca^{2+}]_i$. The Na^+-sensitive Ca^{2+} reservoir was not sensitive to caffeine as illustrated in Fig. 4*C*. In these experiments the nerve endings were loaded with $^{45}Ca^{2+}$ and efflux was studied by perfusing the nerve endings with NM. Addition of caffeine did not induce a rise in $^{45}Ca^{2+}$ efflux whereas perfusion with Na^+ medium triggered a significant increase in the outflow of the radioactive cation. Furthermore, ryanodine (1 μM) which is known to block Ca^{2+}-induced Ca^{2+} release (Fill & Coranado, 1988) did not impair the increase of AVP secretion induced by elevation of the extracellular Na^+ concentration. The amplitude of the Na^+-induced AVP release following addition of ryanodine (S_2) was similar to that of the initial Na^+ application (S_1; $S_2/S_1 = 0{\cdot}9 \pm 0{\cdot}1$, $n = 4$). In summary, the results show that Na^+ can produce a small rise in $[Ca^{2+}]_i$ but this increase cannot account for the amplitude of the Na^+-induced AVP release.

Specificity of cations and anions in inducing AVP release

To determine the specificity for Na^+ of the large increase in release, nerve endings were challenged with a number of cations and anions and secretory responses compared to those obtained to NaCl. In these experiments mannitol, a non-ionic species, was substituted for NMG^+ in the NM. The nerve endings were perfused for 45 min with this medium and then challenged for a period of 32 min with a saline in which mannitol was replaced by either different cation chloride salts or by Na^+ salts (i.e. differing anions). After returning to mannitol-containing medium the nerve endings were perfused with Na^+ medium. The amount of AVP release induced by the first stimulus (S_1) was normalized to second challenge with Na^+ medium (S_2). The *selectivities* of cations and anions for inducing neurohormone release are illustrated in Fig. 5 and are expressed as the ratio S_1/S_2. Among all the monovalent cations tested Na^+ was the most potent cation for inducing the secretory response. K^+ and Li^+ were about half as effective as Na^+ and the decrease in potency of other cations was correlated to their atomic mass. The selectivity among monovalent cations followed series IX of the Eisenman sequence for equilibrium ion exchange: $Na^+ \gg K^+ \geqslant Li^+ > TMA^+ \geqslant Rb^+ \gg Cs^+ > NMG^+$ (Fig. 5*A*). The selectivity for anions was not as straightforward as for cations. However, from the results illustrated in Fig. 5*B* one

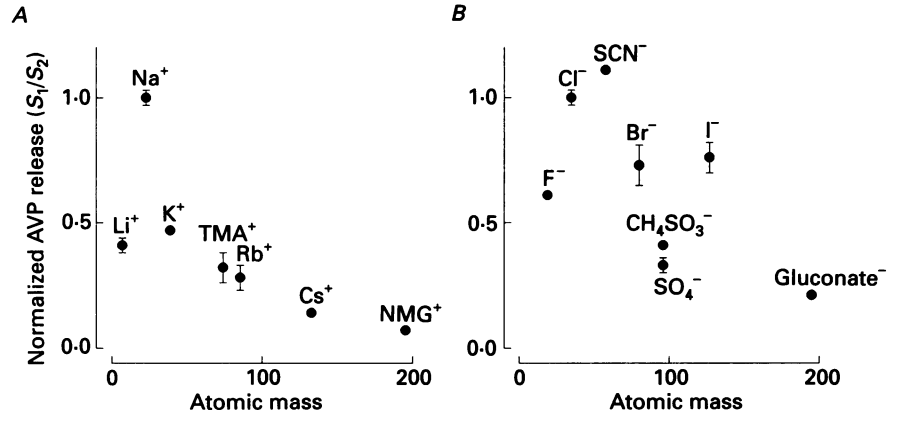

Fig. 5. Specificity of cations (*A*) and anions (*B*) in inducing AVP release. Following perfusion with NM, in which NMG-Cl had been replaced by mannitol, the nerve endings were challenged for 40 min with 140 mM cation chloride salts (*A*) or sodium anion salts (*B*) by equivalent substitution for mannitol. After return to NM–mannitol medium the preparation was perfused for a period of 40 min with Na^+ medium. The results are expressed as the ratio of the amount of AVP release during the first challenge (S_1) to that secreted during the perfusion with Na^+ medium (S_2). The results are given as means ± S.E.M. ($3 \leqslant n \leqslant 12$). Abbreviations are: TMA^+, trimethylamine; NMG^+, *N*-methyl-D-glucamine, $CH_4SO_3^-$, methanesulphonate; SCN^-, thiocyanate.

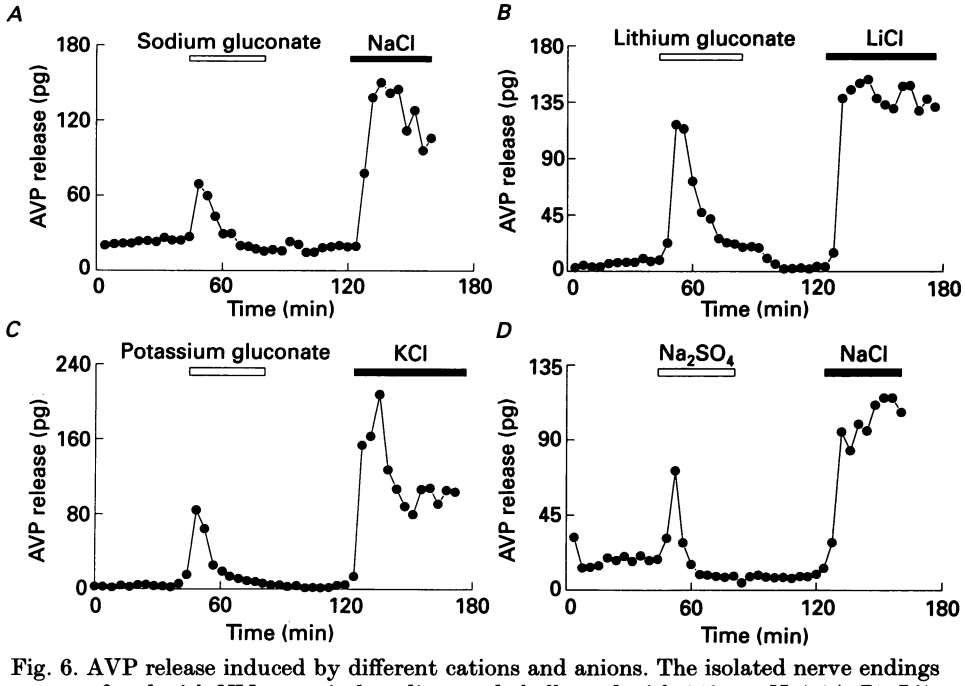

Fig. 6. AVP release induced by different cations and anions. The isolated nerve endings were perfused with NM–mannitol medium and challenged with 140 mM Na^+-(*A*, *D*), Li^+-(*B*) or K^+- (*C*) containing solutions. As indicated by the open bars the cations were associated with either gluconate (*A*, *B*, *C*) or sulphate (*D*) during the first challenge. After return to NM–mannitol medium the nerve endings were challenged with NaCl medium (*A*, *D*), LiCl medium (*B*) or KCl medium (*C*).

can see that Cl^- is also important in promoting secretion. The selectivity for anions was: $SCN^- > Cl^- \gg I^- \geqslant Br^- > F^- \gg CH_4SO_3^- > SO_4^{2} >$ gluconate$^-$ (Fig. 5*B*). Of interest is the observation that, in the presence of a large anion, secretion induced by Na^+, K^+ and Li^+ was of smaller amplitude than in the presence of chloride ions and that the secretory response was *transient*. These results are illustrated in Fig. 6.

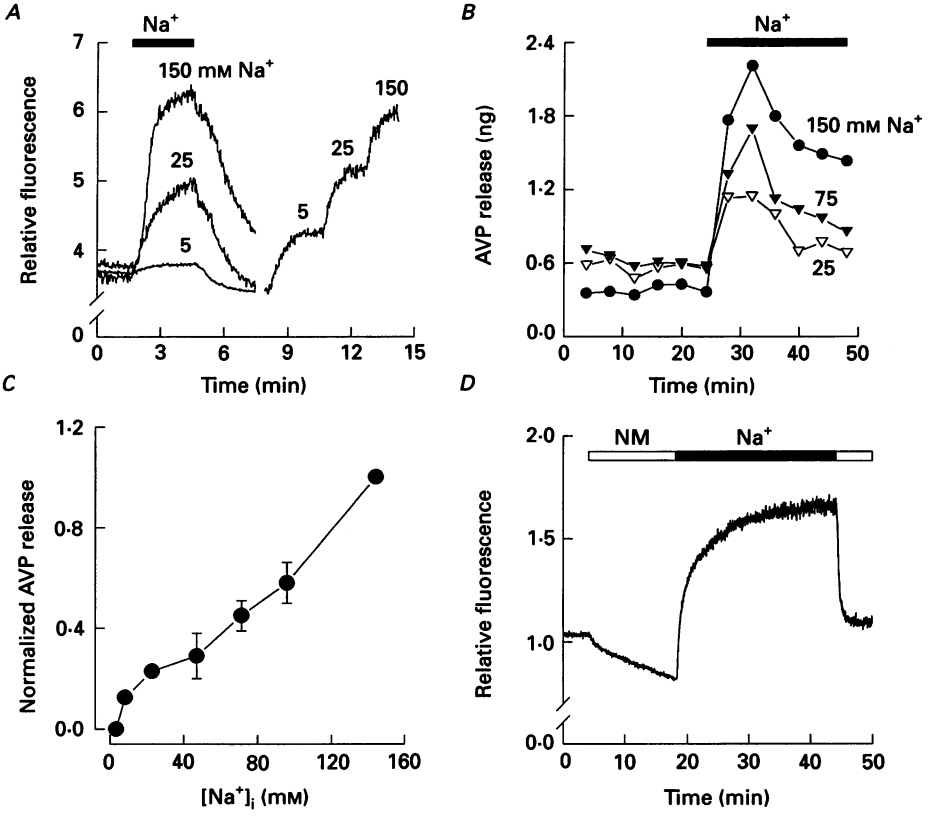

Fig. 7. Effects of $[Na^+]_o$ on $[Na^+]_i$ and AVP release. *A*, changes in relative fluorescence of SBFI-loaded nerve endings perfused in NM and challenged with different $[Na^+]_o$ (mM). After return to NM monensin (10 μM) was added to the medium and calibration was performed by addition of different Na^+ concentrations (mM) as indicated. *B*, AVP release from isolated nerve endings perfused with NM and challenged with different Na^+ concentrations. *C*, relationship between $[Na^+]_i$ and AVP release in isolated nerve endings. The Na^+ concentrations were calculated from experiments similar to those described in *A*. AVP release was normalized by taking as unity the amount of vasopressin release induced by challenging the nerve endings with 150 mM Na^+; the results are given as means $\pm$ S.E.M. ($3 \leqslant n \leqslant 20$). *D*, relative fluorescence of SBFI-loaded nerve endings perfused with Locke solution followed by NM as indicated. Na^+ was substituted for NMG^+ during 27 min as indicated.

Is the Na^+-dependent secretory response related to alteration of the $[Na^+]_i$?

As transitions from NM to Na^+ medium did not result in a correlation between $[Ca^{2+}]_i$ and the AVP secretory response we therefore sought if there was any relationship between the external Na^+ concentration and the extent of AVP

secretion. Figure 7*A* shows results from experiments monitoring the internal sodium concentration ($[Na^+]_i$) of single isolated nerve endings using the fluorescent Na^+ indicator SBFI. Calibration of the responses using monensin showed that challenging the nerve endings with Na^+ medium, following perfusion with NM, gave rise to a

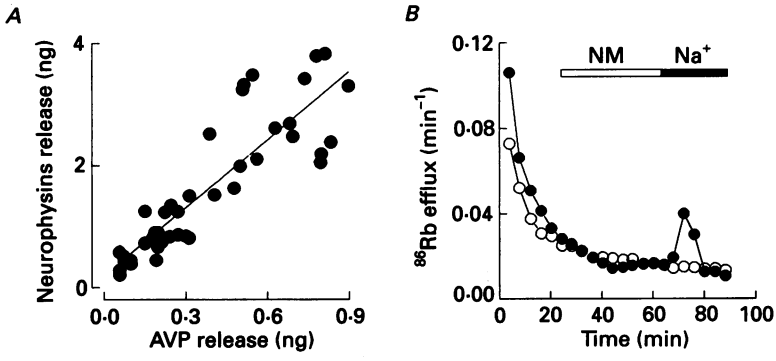

Fig. 8. Release of AVP and neurophysins (*A*) and $^{86}Rb^+$ efflux (*B*) from isolated nerve endings. The amounts of AVP and total neurophysins in the fractions of the perfusate from six independent experiments, in which the nerve endings, after perfusion in NM, were challenged with Na^+ medium, were measured by radioimmunoassay. The data fit a regression algorithm with coefficient of 0·86. *B*, $^{86}Rb^+$-loaded nerve endings were perfused for 20 min in Locke saline and then with NM. In one series of experiments the nerve endings were challenged with Na^+ medium (●) whereas in control experiments (○) the nerve terminals were perfused with NM only. The results are expressed as rate constants and the values are the means of three experiments (the S.E.M. during the perfusion in NM and Na^+ medium were smaller than the size of the symbols).

$[Na^+]_i$ about equal to that of the external $[Na^+]$. Figure 7*B* shows that challenging the nerve endings with different Na^+ concentrations also gave rise to a dose-dependent secretory response. The correlation between the amount of AVP release and the $[Na^+]_i$ is illustrated in Fig. 7*C*. Note that the $[Na^+]_i$ remained elevated during the entire period of Na^+ perfusion and returned towards its original value when the perfusion was switched back to NM (Fig. 7*D*). Therefore, unexpectedly, the data of Fig. 7 demonstrate that the Na^+-induced secretory response is closely correlated to the induced change in $[Na^+]_i$.

Evidence that Na^+-induced secretion occurs by exocytosis

To determine if the Na^+-induced AVP release occurs via exocytosis, we measured the amount of AVP and neurophysins (an approximately 12 kDa molecule originating from the same precursor as AVP and also contained in neurosecretory granules) in the perfusate. The results in Fig. 8*A* show a linear relationship between two intragranular components in the perfusion medium. This type of result has been taken as evidence for exocytotic release in studies on depolarization-induced, Ca^{2+}-dependent secretion. Although a similar relationship may result from lysis of the nerve endings and the granules this is unlikely as (i) no decrease in intensity of the emitted fluorescence associated with the individual excitation wavelengths of fura-2 was observed and (ii) secretory responses of similar amplitude could be induced by

successive Na^+ challenges (Fig. 1*B*). In addition, efflux studies from $^{86}Rb^+$-loaded isolated nerve endings showed no increase in efflux during perfusion with NM and showed, as expected, a transient increase during the early part of the perfusion with Na^+-medium (Fig. 8*B*). This increase may reflect a passive exchange of K^+ with Na^+-ions, required to maintain electroneutrality.

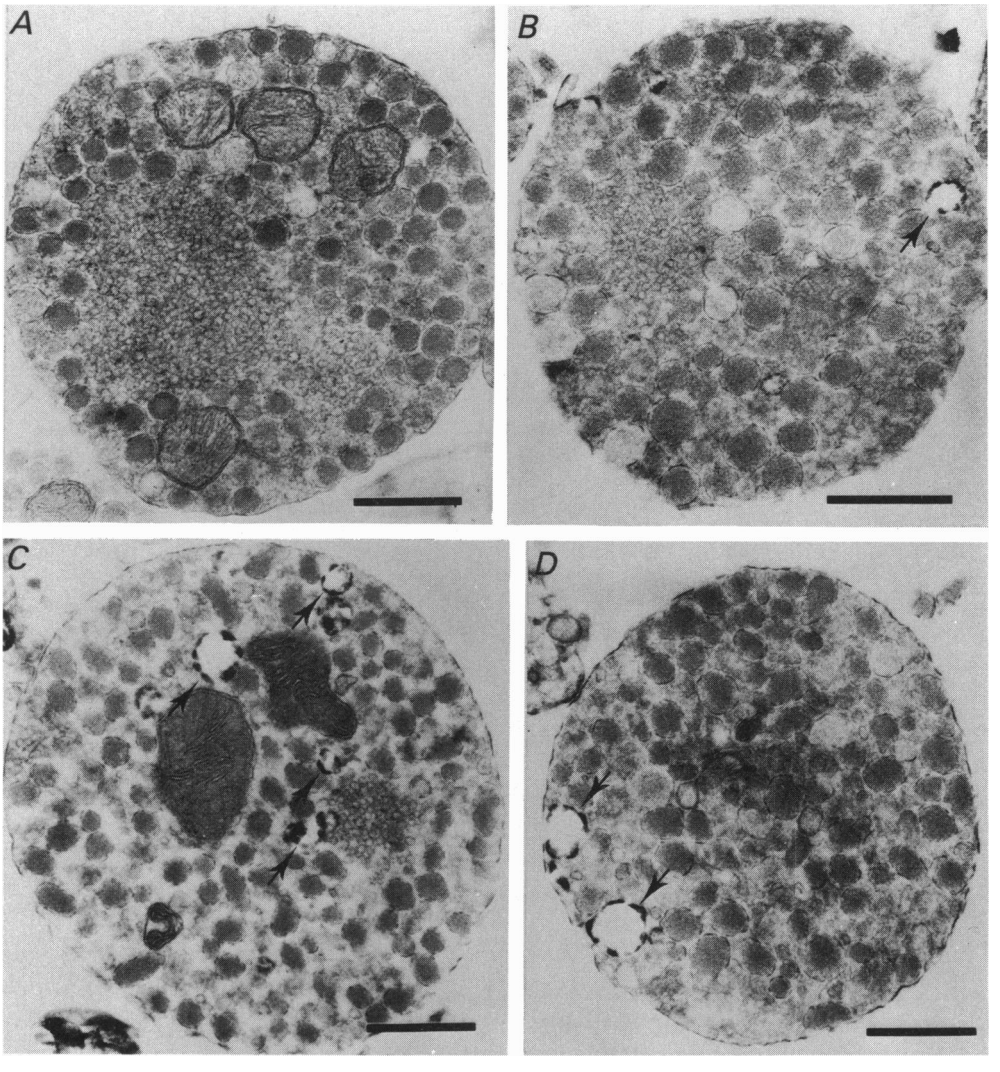

Fig. 9. Electron micrographs of nerve endings perfused in NM (*A*) or Na^+ medium (*B–D*) containing horseradish peroxidase. Note the presence of labelled endocytotic vacuoles (arrows) in the nerve endings undergoing transition from NM to Na^+ medium. Bar, 1 μm.

Additional evidence was obtained from experiments on isolated nerve endings performed in the presence of the fluid phase marker horseradish peroxidase (HRP). Electron micrographs of nerve endings undergoing the transition from NM to Na^+-

medium showed the typical HRP-labelled endocytotic vacuoles associated, in the neural lobe, with the process of exocytosis and endocytosis (Fig. 9). Furthermore, the cytoplasm of the nerve endings showed no labelling suggesting that the nerve endings under these ionic conditions were not permeable to relatively large molecules such as HRP.

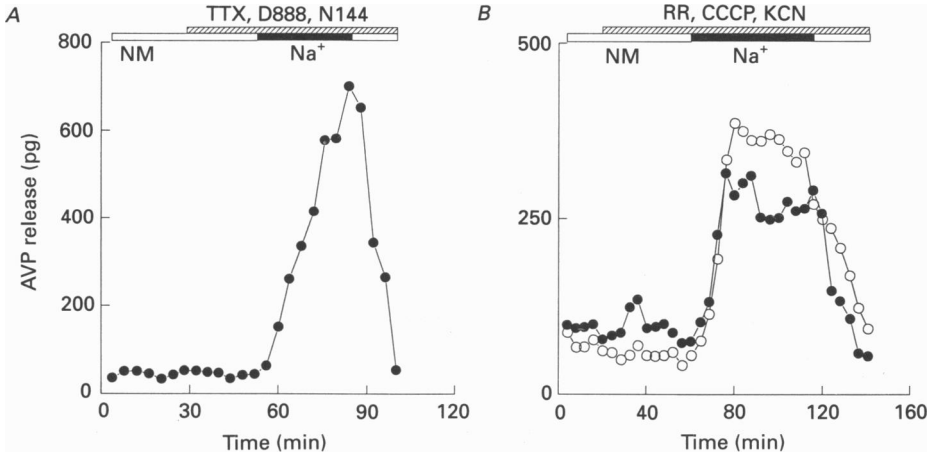

Fig. 10. Effects of channel blockers and metabolic inhibitors on the release of AVP from isolated nerve terminals. The nerve endings were perfused with NM and Na^+ medium as indicated. *A*, the channels blockers TTX (1 μM), D888 (10 μM) and N144 (10 μM) were added to the media as indicated by the hatched bar. *B*, the metabolic inhibitors Ruthenium Red (RR, 100 μM), KCN (400 μM) and the uncoupler CCCP (10 μM) were added as indicated. The results (●) are compared with control experiments (○).

Effects of channel blockers and metabolic inhibitors on the Na^+-induced secretory response

The results from the fluorescent indicators demonstrated that considerable ionic redistribution does occur upon transition from NM to Na^+- or Ca^{2+}-containing medium. We, therefore, tried to determine if some of the ionic channels characterized from these nerve endings could be pathways involved in the redistribution of Na^+, Ca^{2+} and Cl^- ions. The inhibitors were added to the NM 20 min before substituting Na^+ for NMG^+. When added independently TTX (tetrodotoxin), D888 (desmethoxyverapamil), N144 (5-nitro-2-(3 phenylpropylamino)-benzoic acid) or SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid), used to block voltage-sensitive Na^+, Ca^{2+} and Cl^- channels, respectively, did not prevent the increase in AVP release induced upon switching from NM to Na^+ medium (not illustrated). Furthermore, TTX, D888 and N144 added together during NM perfusion did not inhibit the subsequent Na^+-dependent AVP release, as illustrated in Fig. 10*A*.

In another series of experiments we used metabolic inhibitors and uncouplers of oxidative phosphorylation to see if the secretory response to Na^+ required a maintained elevated energy charge. The results are shown in Fig. 10*B* and demonstrate that in the presence of Ruthenium Red (RR), carbonyl cyanide *m*-

chlorophenylhydrazone (CCCP) and potassium cyanide (KCN) Na^+ could still induce a large release of AVP corresponding to 83% of that seen in untreated endings from the same preparation. Note that at the onset of the perfusion with the medium containing the inhibitors there was a small increase in secretion perhaps reflecting the release of Ca^{2+} from internal reservoirs.

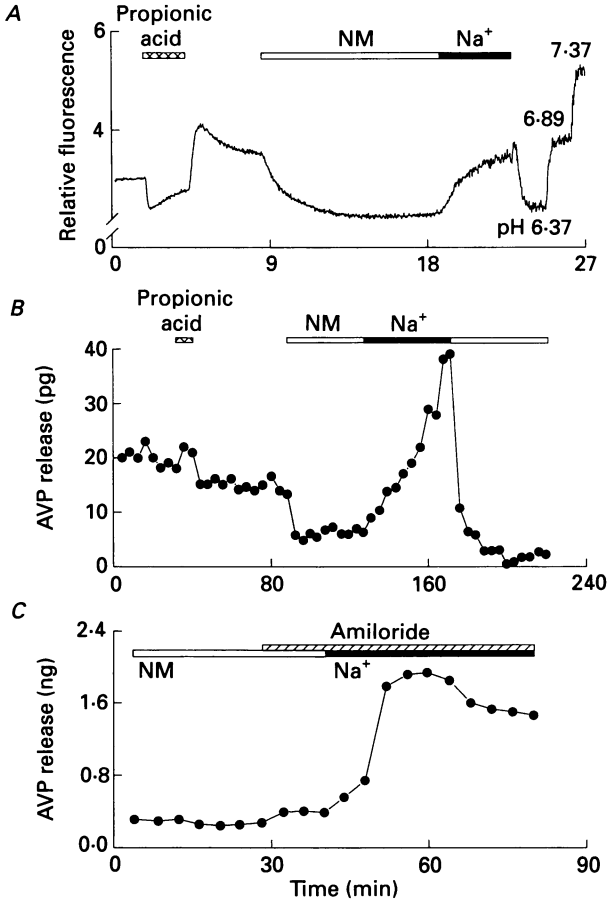

Fig. 11. Effects of alteration of internal pH on AVP release. *A*, pH_i in a single nerve ending preloaded with the pH indicator BCECF. The nerve endings were first perfused with Locke solution and, as indicated, 50 mM sodium propionate was substituted for NaCl. After return to Locke solution the nerve endings were perfused with NM and then with Na^+ medium. Calibration was performed as described in Methods. *B*, AVP release from isolated nerve endings perfused with salines using the same protocol as that described in *A*. *C*, nerve endings perfused with NM (open bar) and Na^+ medium (filled bar) to which the blocker of the Na^+–H^+ exchanger amiloride (1 mM) was added as indicated.

Effects of internal pH on the secretory response

The ubiquitous presence of a Na^+–H^+ exchanger in plasma membranes suggests that complete removal and replacement of external Na^+ may lead to changes in intracellular pH. In order to determine if the increased AVP secretion upon a Na^+

challenge could result solely from a change in intracellular pH (pH_i) we used the pH-sensitive fluorescent indicator BCECF to analyse the variations of pH_i induced by the transition from NM to Na^+ medium. Figure 11*A* shows that perfusion of the isolated nerve endings with NM induced a significant drop of pH_i, as expected by

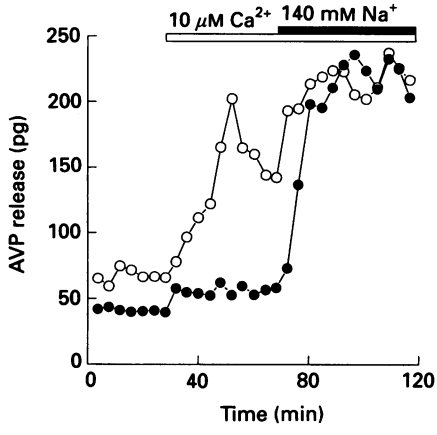

Fig. 12. Effects of $[Ca^{2+}]_i$ buffering on the Na^+-induced AVP release. The nerve endings were preloaded with BAPTA (●) and perfused with NM. Ca^{2+} (10 μM) was first added followed by Na^+ (140 mM) substitution for NMG^+ as indicated. The results are compared to those of control (i.e. unloaded) nerve endings.

reverse operation of the Na^+–H^+ exchanger, which could be reversed by substituting Na^+ for NMG^+. This drop in pH_i could be mimicked by the addition of propionic acid (50 mM) to a normal Locke saline. We therefore investigated, using propionic acid, if such a fall and recovery in pH_i *per se* could explain the large and sustained Na^+-dependent secretory response described above. The results are presented in Fig. 11*B* and show that neither the reduction in pH_i induced by propionic acid nor the transient alkalinization upon removal of propionic acid produced a change in the amount of AVP release. In the same experiments, however, a large increase in the secretory response was observed following the NM–Na^+ medium treatment (Fig. 11*B*). In additional experiments nerve endings were perfused with NM containing amiloride, a blocker of the Na^+–H^+ exchanger. The results are illustrated in Fig. 11*C* and show that amiloride did not block the effect of Na^+ in inducing a large release of AVP. Similar data were obtained in five experiments. Altogether these results demonstrate that modification of pH_i does not affect the secretory response triggered by substituting Na^+ for NMG^+, it should be noted that the resting pH_i in the intact nerve endings was routinely found to be more acidic ($\leqslant$ pH 6·9) than expected. This may result from a portion of fluorescent signal arising from indicator within the acidic neurosecretory granules.

Na^+ induces the release of AVP from highly Ca^{2+}-buffered nerve endings

Although correlation of changes in $[Ca^{2+}]_i$ to the Na^+-induced secretory response seemed unlikely, based on the preceding experiments, it remained possible that locally high Ca^{2+} concentrations may be generated near sites of Ca^{2+} release from the

stores. Locally high Ca^{2+} concentrations would be underestimated as a result of the Ca^{2+} monitoring approach used here which averages the $[Ca^{2+}]_i$ over the entire terminal. Thus to examine if the Na^+-dependent AVP release could result from such a local phenomenon we used dimethyl-BAPTA (1,2-bis(*O*-aminophenoxy)ethane-

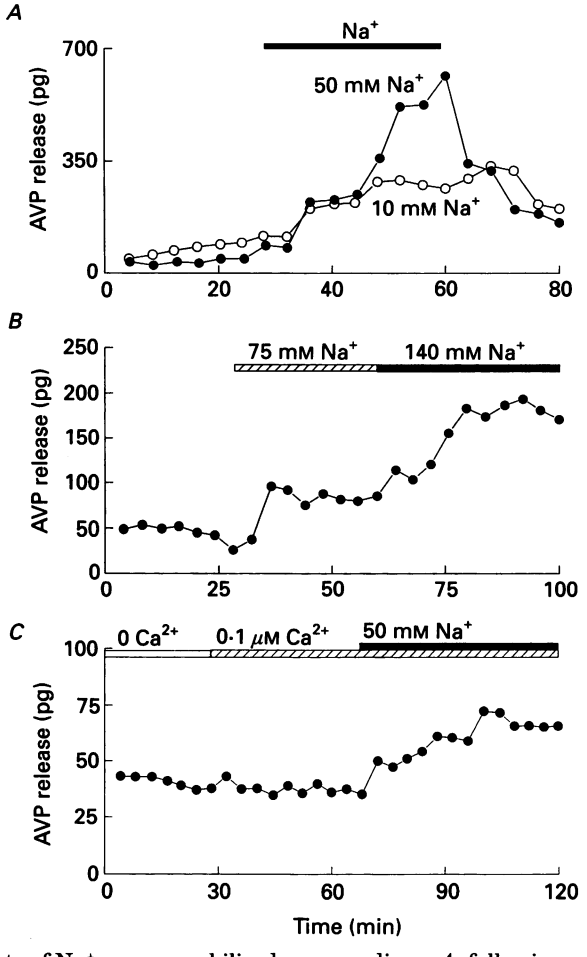

Fig. 13. Effects of Na^+ on permeabilized nerve endings. *A*, following permeabilization with streptolysin-O, the nerve endings were perfused with Ca^{2+}-free, potassium glutamate containing medium and Na^+ (10 mM, ○; 50 mM, ●) was substituted for K^+ as indicated. *B*, following permeabilization with digitonin the nerve endings were perfused with Ca^{2+}-free, potassium glutamate-containing medium. 75 mM (hatched bar) and 150 mM (filled bar) Na^+ were substituted for K^+ as indicated. *C*, following digitonin permeabilization nerve endings were exposed to 0·1 μM free Ca^{2+} followed by addition of 50 mM Na^+ substituted for NMG^+.

N,N,N′,N′-tetraacetic acid)-loaded, intact, nerve endings. BAPTA exhibits a much greater rate of Ca^{2+} binding than alternative buffers such as EGTA (Augustine, Adler & Charlton, 1991), and as shown previously, result in almost total abolition of the Ca^{2+}-dependent K^+-induced AVP secretion (Stuenkel & Nordmann, 1993). The

results are illustrated in Fig. 12 and show that after being loaded with BAPTA isolated nerve endings were still fully responsive to a Na^+ challenge (112% of control), but not Ca^{2+}, further demonstrating that the Na^+-dependent AVP release described in the present paper is Ca^{2+} independent.

Effects of $[Na^+]_i$ on permeabilized nerve endings

The experiments described above were performed on intact, isolated nerve endings in which ionic redistribution could be induced as a result of perfusion with NM (see Figs 2 and 7). As an alternative approach to alter directly the ionic conditions of the terminal cytoplasm we permeabilized the nerve endings with digitonin or streptolysin-O. This approach allowed direct, unimpeded diffusion of ions through the plasma membrane and to equilibration of the intraterminal milieu with the extracellular medium. Figure 13*A* illustrates the results of experiments in which the K^+ concentration was kept constant at 90 mM, the osmolarity of the medium being maintained with NMG glutamate. After permeabilization the nerve endings were challenged with 10 or 50 mM sodium glutamate. This led to a secretory response which was dose dependent. Figure 13*B* shows that substituting 75 mM Na^+ for K^+, 60 min after digitonin permeabilization induced an increase in AVP release which was further increased by complete substitution by Na^+ for all the K^+. Furthermore, complete substitution of Na^+ for K^+ led to a secretory response ($7{\cdot}8 \pm 0{\cdot}35$-fold at peak increase, $n = 8$) similar to that observed to Na^+ challenge on the intact nerve endings. Finally, we performed experiments in which the effects of Na^+ were tested in the continued presence of 0·1 μM free Ca^{2+}, a basal calcium level similar to that found in intact nerve endings (Fig. 13*C*). Nerve endings were initially permeabilized with digitonin in the absence of Ca^{2+}. The free Ca^{2+} concentration was then increased to 0·1 μM with a medium containing (mM): potassium glutamate, 140; Tris-Pipes (piperazine-*N*-*N*′-bis(2-ethanesulphonic acid), 10, pH 6·8; EGTA, 2; $MgCl_2$, 1; $CaCl_2$, 0·4; glucose, 10; BSA, 0·01%. following a 40 min incubation the Na^+ concentration was increased to 50 mM, the $[K^+]$ concentration being reduced accordingly. This resulted in an increase in AVP secretion similar to those described above.

DISCUSSION

In this paper we present evidence for a direct correlation between the internal Na^+ concentration and the release of AVP from isolated rat neurohypophysial nerve endings. This is supported by the direct observation in single nerve endings of rapid, reversible and dose-dependent changes in $[Na^+]_i$ that correlate with the transition from NM to Na^+ medium. In addition, the time course of the change in $[Na^+]_i$, together with the ability to repetitively evoke these changes, compared closely with the secretory responses induced to Na^+ medium. This is in striking contrast to the observed changes in intracellular Ca^{2+} under identical experimental protocols. While Na^+ medium was capable of evoking changes in $[Ca^{2+}]_i$ by release from intracellular stores, these were of small amplitude, transient and declined in amplitude with repetitive Na^+ challenges, properties in contrast to the secretory response.

Explanation of the secretory response by Na^+-induced changes in Ca^{2+} are unlikely to be based on: (i) the size of the secretory response to the change in averaged $[Ca^{2+}]_i$

produced by $[Na^+]_i$ relative to the amount of AVP release and change in averaged $[Ca^{2+}]_i$ by elevated K^+; (ii) the competence of the BAPTA-loaded nerve endings to give a secretory response to challenge with external Na^+; (iii) the observation that permeabilized nerve endings dose-dependently respond by secretion of AVP in response to elevation of Na^+ in the presence of the calcium chelator EGTA. Although it is difficult to entirely exclude the possibility of Ca^{2+} as a mediator of the Na^+-evoked secretory response based on the possibility of locally high Ca^{2+} concentrations generated near sites of exocytosis, the multiple approaches utilized strongly suggest such a scenario is not reasonable.

An important consideration for the understanding of the Na^+-induced secretory response is whether it occurs as the result of a normal exocytotic process. The evidence presented relating the relationship between AVP and neurophysins release and the demonstration of endocytotic vacuoles by HRP labelling suggests that Na^+-induced secretion occurs via exocytosis. This is further substantiated by the lack of leakage from the nerve endings of fura-2 following perfusion with NM and Na^+ medium. The Na^+-evoked exocytosis of vasopressin argues against participation of a Na^+-coupled transport process similar to that described for some neurotransmitters. Although we do not yet understand how the ionic redistribution occurs on transition from NM to Na^+ medium, the conclusion that Na^+ exerts a direct effect on secretion is of greater importance at present than the mechanism by which the redistribution occurs. It is of importance to note that a change in the secretory response of neurohypophysial nerve endings also occurs following changes in $[Na^+]_o$ in normal saline (Toescu & Nordmann, 1991), demonstrating that the treatment with NM is not a prerequisite for a Na^+-induced secretory response to occur.

A possible explanation of the Na^+-induced secretory response is that, under the experimental conditions utilized where Ca^{2+} concentrations have been kept low, Na^+ is exerting its action by binding to the Ca^{2+} 'receptor sites' for exocytosis. By analogy, studies on the selectivity of Ca^{2+} channels show that chelation of external Ca^{2+} alters the channel selectivity to allow considerable Na^+ permeability (Kostyuk & Krishtal, 1977; Almers, McCleskey & Palade, 1984). In addition, evidence has been presented that heavy metals such as Sr^{2+} and Ba^{2+} can evoke considerable secretory responses presumably by direct substitution for Ca^{2+} at appropriate binding sites (Zengel & Magleby, 1981). The Na^+ effects on secretion presented here may, in part, be accounted for by such an action. However, the maintained secretory response evoked by Na^+ relative to the transient effect of Ca^{2+} suggests that additional actions are present. It is as if Ca^{2+} could only induce the release of a small immediately releasable pool of neurosecretory granules, whereas Na^+ could additionally promote the release of a larger population of granules. The dramatic difference in the time course of the Ca^{2+}-dependent release ($t_{\frac{1}{2}} = 45$ s; Stuenkel & Nordmann, 1993) compared to that of the Na^+-dependent secretory response ($t_{\frac{1}{2}} > 1$ h, see Fig. 1*A*) occurs even under conditions where both Ca^{2+} and Na^+ are maintained at elevated cytosolic concentrations. Moreover, the disparity of the secretory response is observed even in permeabilized nerve endings where local differences in concentration gradients cannot be invoked to explain the differences in the secretory response. A further important observation is presented in Fig. 2 where, following perfusion with NM, Ca^{2+} gives rise to a reduced AVP secretory response, when added to the medium

after the Na^+. In contrast, a secretory response can be induced by Na^+ irrespective of the order with which the cations are added to the medium. These data suggest, surprisingly, that Ca^{2+} can release only a fraction of the granules sensitive to Na^+. A puzzling result, related to the duration of the secretory response, was the finding that in the presence of Cl^-, irrespective of the monovalent cation, the secretory response was prolonged (Fig. 6) whereas in the presence of other anions (e.g. SO_4^{2-}), irrespective of the cation, release was transient. This suggests that the nature of the anion might be of importance for maintenance of secretion (Dayanithi & Nordmann, 1989). Note, however, that in the presence of $CaCl_2$ the secretory response was *always* transient. This suggests that a rise in $[Ca^{2+}]_i$ can initially be stimulatory while, in the presence of its continued rise, is unable to maintain secretion (see Stuenkel & Nordmann, 1993).

One of the possible effects of Na^+ on the secretory response could be related to osmotic effects. Changes in osmotic pressure have been shown to promote or decrease fusion of secretory granules (Pollard, Tack-Goldman, Pazoles, Creutz & Shulman, 1977; Cohen, Akabas & Finkelstein, 1982) although recent studies have demonstrated that swelling of the granule is not a prerequisite for membrane fusion to occur (Zimmerberg, Curran, Cohen & Brodwick, 1987). In the present study a number of observations suggest that the secretory response is not directly related to osmotic effects on the secretory granules or the nerve endings. These include: (i) a selectivity for the induced release that is not in direct proportion to the expected permeability based on size of the cation involved (i.e. Li^+ being less selective than Na^+); (ii) the finding of a secretory response in permeabilized nerve endings to 10 mM Na^+ under conditions where osmotic effects can be considered negligible (see Fig. 13).

In the present paper we present strong evidence for a Ca^{2+}-independent mechanism of neurosecretion. A role for Na^+ in the modulation of release of neurotransmitter, especially as related to synaptic facilitation and post-tetanic potentiation, has been reported by various groups (Gage & Quastel, 1965, 1966; Birks & Cohen, 1968*a*, *b*; Muchnick & Venosa, 1969; Baker & Crawford, 1975; Statham & Duncan, 1977; Charlton, Thomson, Atwood & Farnell, 1980). The involvement of Na^+ was attributed to an indirect role in each of these studies, via its presumed effects on $[Ca^{2+}]_i$ or on the exocytotic Ca^{2+} receptor. However, using ionic substitution studies and monitoring of $[Ca^{2+}]_i$ and $[Na^+]_i$ we show here that Na^+ can exert a direct, Ca^{2+}-independent, effect on secretion. Moreover the increase in $[Na^+]_i$ necessary to trigger release is within the physiological range expected to elevated neuronal electrical activity. Direct measurement of $[Ca^{2+}]_i$ and $[Na^+]_i$ in nerve endings of neurons after tetanic stimulation is presently being investigated to confirm our unorthodox findings.

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