Ca\textsuperscript{2+}-independent regulation of neurosecretion by intracellular Na\textsuperscript{+}

Jean J. Nordmann\textsuperscript{1} and Edward L. Stuenkel\textsuperscript{2}

\textsuperscript{1}Centre de Neurochimie CNRS, 5 rue Blaise Pascal, 67084 Strasbourg Cedex, France and \textsuperscript{2}Department of Physiology, 7744 Medical Sciences II Building, University of Michigan, Ann Arbor, MI 48109, USA

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While secretion from nerve endings is strictly controlled by an increase in cytoplasmic free calcium several reports suggest intracellular sodium may serve a regulatory role. Whether sodium acts directly to modulate secretion or indirectly by influencing cytoplasmic calcium dynamics is unknown. This study shows, based on parallel experiments studying [Na\textsuperscript{+}], [Ca\textsuperscript{2+}] and vasopressin secretion, that sodium acts directly to regulate secretion in isolated nerve endings from the rat neurohypophysis. The elevation in secretion that develops is dose-dependently related to the [Na\textsuperscript{+}], and can occur in the absence of changes in [Ca\textsuperscript{2+}].

1. INTRODUCTION

A change in the free intracellular calcium concentration in nerve endings is almost universally accepted as the major regulator of exocytosis [1]. However, in a number of neuronal preparations, prolonged high-frequency stimulation results in a potentiation of neurosecretion [2-6] that has been linked to increased intracellular sodium. In each case, the sodium-dependent potentiation of release has been attributed to either sodium-induced calcium release from intracellular stores [4,7,8] or to enhancement of sodium-calcium exchange [9-11]. Until now, little or no attention has been paid to the possibility of a direct action of sodium on exocytosis. To investigate this possibility we have monitored the intracellular sodium and calcium concentrations together with the release of vasopressin (AVP) from isolated rat neurohypophyseal nerve endings. The results suggest that there is a direct effect of intracellular sodium on exocytosis that is independent of calcium. It is concluded that Na\textsuperscript{+} per se may be an intrinsic regulator of basal neurosecretion.

2. MATERIALS AND METHODS

The isolated nerve endings were prepared according to [12]. Briefly, rat neurohypophyses were separated from the pars intermedia and homogenized in a solution containing (mM): sucrose 270, HEPES-KOH 10 (pH 7.0), EGTA 2. The homogenate was centrifuged at 100 \times g for 1 min and the supernatant was further centrifuged at 2400 \times g for 4 min. The resulting pellet contains highly purified nerve endings from both AVP and oxytocin neurons [13]. The nerve endings were loaded onto filters (0.22 \mu m Acro disk, Gelman) and perfused at 37°C

Measurements of [Ca\textsuperscript{2+}] and [Na\textsuperscript{+}], were performed on single nerve endings by dual-wavelength microspectrofluorimetry of fura-2 and SBFI, respectively, loaded into the nerve endings [15]. For fura-2 loading, nerve endings were incubated with 1.5 \mu M fura-2/AM for 20 min at 37°C in normal physiological saline. Nerve endings loaded with SBFI were incubated with 15 \mu M SBFI/AM for 40 min. The SBFI/AM stock solution was prepared to a final concentration of 5 mM in DMSO with 12.5% pluronic acid. Following loading nerve endings were centrifuged (4 min at 2400 \times g) and resuspended in normal physiological saline. All [Ca\textsuperscript{2+}] and [Na\textsuperscript{+}] measurements were performed at 37°C on isolated endings as described previously [15] which naturally adhered to a coverslip forming the bottom of a chamber (vol. 100 \mu l) which was maintained under constant perfusion (1.5 mm/min). Individual endings were viewed on a Nikon Diaphot inverted microscope equipped with a 40\times (1.4 numerical aperture) oil immersion, epifluorescent lens and optically isolated using a pinhole diaphragm. Monitoring of [Ca\textsuperscript{2+}] and [Na\textsuperscript{+}], was performed using SPEX fluorolog and SPEX ACRM systems by monitoring the emitted, fluorescent, photon counts at 510 nm at each excitation wavelength (340 nm and 380 nm). For Ca\textsuperscript{2+} measurements the fluorescence ratio (340 nm : 380 nm) was converted to [Ca\textsuperscript{2+}], using the equation of Grynkiewicz et al. [16] and a value of 224 nM for the K\textsubscript{d} of fura-2. Values of R\textsubscript{min}, R\textsubscript{max} and F/Fo were determined using an external standard calibration technique. Calibration of the RF (340 nm : 380 nm) to [Na\textsuperscript{+}], in the SBFI experiments was performed using the Na\textsuperscript{+} with saline [12]. The perfusate (4 min periods) was collected 1 h after loading and evoked release was triggered by increasing, during 8 min, the extracellular K\textsuperscript{+} concentration (50 mM). AVP in the perfusate was determined by radioimmunoassay [14]. The normal physiological saline contained (mM): NaCl, 40; KHCO\textsubscript{3}, 5; N-methyl-D-glucamine- (NMG) 100; MgCl\textsubscript{2}, 1; CaCl\textsubscript{2}, 2.2; glucose, 10; HEPES-Tris, 10 (pH 7.0-7.2); BSA, 0.01%. When the external K\textsuperscript{+} concentration was increased the NaCl concentration was reduced accordingly in order to maintain the osmolarity of the perfusion medium at a constant 305-310 mOsm. The nerve endings were then perfused with NM containing (mM): N-methyl-D-glucamine-CI, 150; EGTA 0.1; Tris-HEPES, 10 (pH 7.0-7.1); glucose, 10; 0.01% BSA. When Na was added, NMG was reduced accordingly. Incubation in NM did not lyse or permeabilize the nerve endings as no increase in leak of the fluorescent probes used to measure ionic activities from that in a more complete physiological saline was observed. In all the experiments reported in this paper great care was taken to adjust the osmolarity of all the media to 305-310 mM.

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ionophore monensin as an internal standard technique. Note that the value for [Na'] at 150 mM [Na+] must be taken with great caution as a result of reduced sensitivity of SBFI at concentrations above 50 mM. In some cases the Na' data is shown as the SBFI fluorescence ratio (340 nm:380 nm; RF). For [3Ca2+] efflux nerve endings were preincubated for 60 min in normal physiological saline containing 10 µCi 40Ca2+. They were then loaded onto filters and perfused in NM. Fractions (200 µl) were collected in 2 min intervals. Efflux was expressed as a rate constant k ((min-1) = ΔX/dt × t, where ΔX represents counts/min of 40Ca2+ released in the mean time interval dt and t, the tissue content at the mid-point of interval dt.

Digitonin-permeabilized nerve endings, when used, were prepared as follows. After loading of the isolated endings on the filter and 45 min perfusion with normal physiological saline they were perfused for 5 min in the same medium but lacking Ca2+ and to which 2 mM EGTA had been added. Perfusion was continued with a saline containing (mM): K-glutamate 140, MgCl2 2, EGTA 2, Tris-Pipes 10 (pH 7.0), glucose 10 and BSA 0.01%. Following permeabilization with digitonin (2 µM, 5 min) the nerve terminals were perfused with the same medium. Fractions were collected 30 min after the permeabilization period and AVP in the perfusate was determined by radioimmunoassay.

3. RESULTS AND DISCUSSION

The role of sodium (Na+) in triggering arginine vasopressin release from isolated rat neurohypophyseal nerve endings was analyzed by ionic substitution studies. All ionic substitutions were performed on nerve endings initially perfused with a minimal medium, termed ‘nothing’ medium (NM, see section 2), that lacked permeant cations. Fig. 1a shows the effect of admission of Na+ alone on secretion of AVP from nerve endings pretreated with NM for 40 min and its comparison to a K+ evoked response in a more complete physiological saline. Sodium induced a robust increase in AVP secretion (7.0 ± 0.8-fold increase above NM alone over a 20 min period, n=28) which exhibited a time course parallel to that of the duration of the Na+ pulse. The effect of Na+ alone, on secretion, was reversible and could be re-established on sequential pulses of Na+ (Fig. 1b). The release of AVP by exocytosis was confirmed by conventional electron microscopy [17] (not shown). Each application of Na+, following a period in NM, resulted in a secretory response which showed slight desensitization from the initial Na+ pulse. Surprisingly, admission of Ca2+ alone, after a Na+ challenge, failed to result in a significant secretory response even though a substantial Na+-induced response could be subsequently produced on the same nerve terminals (Fig. 1c). These latter results suggest selectivity of the secretory response among applied cations. Analysis of the selectivity for monovalent cations was found to follow series IX of the Eisenman sequence for equilibrium ion exchange [18] (Na+ >K+ >Li+ >Rb+ >Cs+; not illustrated). Similar experiments performed in NM containing 3.2 mM Mg2+ did not prevent the effect of addition of external Na+ on secretion suggesting that the physiological divalent cation concentration at the outer surface of the membrane does not alter the Na+ effect.

One plausible explanation of the Na+ effect on AVP release is that its application to NM treated nerve terminals generates a rise in intracellular Na+ ([Na+]i) which then acts to mobilize Ca2+ from internal stores. We therefore examined, using ion-sensitive fluorescent probes [16,19,20] the effect of Na+ or Ca2+ addition on the intracellular Na+ and Ca2+ concentrations. Furthermore, unidirectional Ca2+ movement was analyzed by monitoring [45Ca2+] efflux from preloaded endings under similar ionic treatments. Fig. 2a shows the effect, following perfusion with NM, of addition of Na+ alone or Na+ and Ca2+ added together on [Ca2+]i in single, isolated nerve terminals. The amplitude of the [Ca2+]i increase is compared with that obtained by depolarizing the nerve endings using a conventional increase of the extracellular K+ concentration in a more complete physiological saline. Whereas, following perfusion with NM, Na+ induced a small increase of 50 ± 11 nM (mean

Fig. 1. Effects of external Na+ and Ca2+ on the release of AVP from nerve endings incubated in ‘nothing’ medium (NM). (a) The nerve endings were incubated in normal physiological saline and evoked AVP release was triggered by increasing the external potassium concentration. The nerve endings were then returned to normal saline before they were perfused with NM. After a period of 40 min Na+ was added, as indicated, at a concentration of 150 mM. (b) The nerve endings were incubated in NM and challenged repetitively with 150 mM Na+ as indicated. (c) The nerve endings in NM medium were challenged first with 150 mM Na+. After a return to NM, calcium was added at a concentration of 2.2 mM. A second Na+ challenge (150 mM) together with Ca2+ (2.2 mM) was given following a 40 min period in NM. In this and all subsequent figures changes in perfusate ionic composition are as indicated below plots.
addition of Ca²⁺ to the extracellular medium induced a large and sustained increase of 244 ± 37 nM (n = 13) in [Ca²⁺]. Monitoring ⁴⁵Ca⁺ efflux showed a significant increase in addition to an initial pulse of Na⁺, suggesting a Na⁺-induced release of Ca²⁺ from internal stores. Therefore the small rise in [Ca²⁺] seen to Na⁺ application in Fig. 2a may result from a Na⁺-dependent increase in intracellular Ca²⁺ mobilization and subsequent efflux. However following recovery in NM, a second sodium pulse induced a considerably reduced ⁴⁵Ca⁺ efflux response (Fig. 2b) and a lack of increase in [Ca²⁺], (not shown). It should be noted that the above experiments present two inconsistencies with the conclusion that the secretory response to Na⁺ challenge results from Ca²⁺ mobilization and/or rise in [Ca²⁺]. These include: (i) the amplitude of the secretory response is much greater than that expected from the rise in [Ca²⁺]; alone (see below); and (ii) the time course of the secretory response induced by a Na⁺ challenge is sustained while that related to Ca²⁺-induced secretion has been shown to be transient [12]. Furthermore, the observation of repetitive secretory responses to Na⁺ application following NM treatment (Fig. 1b) in the absence of significant Ca²⁺ responses suggests a dichotomy between the secretory response and [Ca²⁺]. To investigate whether the Na⁺-induction of AVP release, following NM treatment, could result from a rise in intracellular Na⁺ concentration, we monitored, using the Na⁺-sensitive, fluorescent probe SBFI, the [Na⁺]; under similar treatments. As shown in Fig. 2c application of Na⁺ to NM-treated nerve endings results in a significant increase in intracellular Na⁺ whose kinetics closely parallel that of the induced secretory response to a Na⁺ challenge. Moreover, repetitive application of extracellular Na⁺ gave rise to an increase in [Na⁺], that showed little variation from the initial Na⁺ pulse (Fig. 2c, inset). The increase in [Na⁺], was unaffected by the presence of the Na⁺ channel blocker tetrodotoxin, as was the secretory response (not shown). The observations of repetitive increases in [Na⁺], and of AVP release to induced challenges of extracellular sodium without pronounced increases of [Ca²⁺], suggest a direct relationship between the changes in [Na⁺], and neurosecretion and, furthermore, confirm that release occurs by exocytosis. It should also be noted that depolarization of the nerve endings with elevated K⁺ in the absence of

Fig. 2. Effects of extracellular Na⁺ on [Ca²⁺], ⁴⁵Ca⁺ efflux and [Na⁺], on isolated nerve endings following NM treatment. (a) Representative comparison of change in [Ca²⁺] induced by a depolarising concentration of elevated K⁺ (50 mM; 30 s) with a pulse of 150 mM Na⁺, or of 150 mM Na⁺ and 2.2 mM Ca²⁺ added together. The latter treatments followed a period in NM while the K⁺ depolarization was performed in normal physiological saline. (b) Representative increase in rate constant of ⁴⁵Ca⁺ efflux upon sequential additions of 150 mM Na⁺ following NM treatment. (c) Effect of addition of extracellular Na⁺ (150 mM) on [Na⁺], in NM-treated nerve endings. A second Na⁺ challenge was performed in the presence of 10° M tetrodotoxin. The ratio of fluorescent values (RF) (340 nm-380 nm) are plotted as a function of time. A, B and C at the end of the plot represent 0 mM, 25 mM and 150 mM Na⁺ in the presence of monensin. The inset shows the effect of repetitive periods of application of Na⁺ (150 mM) on [Na⁺], which are preceded by NM treatment.

Fig. 3. Relationship between [Na⁺], [Na⁺], and AVP release in NM-pretreated nerve endings. ● [Na⁺]; ○, normalized AVP release; ▼, resting [Na⁺] measured under normal physiological saline. The results are given as mean ± SEM. The inset shows a portion of the above relationship on an expanded scale. Normalized AVP release was calculated by comparing the secretory response at a given [Na⁺], against that obtained at 150 mM [Na⁺]. Note that due to the lack of sensitivity of SBFI at high [Na⁺] the obtained value of [Na⁺], at a [Na⁺], of 150 mM is higher than expected (parentheses). All values are expressed as mean ± SEM with 3<n<20.
extracellular Ca$^{2+}$ does not result in a secretory response [21]. Therefore, its induction to application of Na$^+$ following NM treatment is not a direct result of membrane depolarization (see also permeabilized nerve ending results below). As perfusion with NM is expected to result in a significant change in intracellular pH we have used the pH sensitive, fluorescent probe BCECF to monitor the intracellular pH. Duplication of these pH changes with propionic acid were ineffective in producing the secretory response observed to a Na$^+$ pulse following NM treatment (not illustrated).

The possibility of a relationship between [Na$^+$], and AVP release led to a closer examination of the relationship over a range of [Na$^+$]. The dose–response relationship between the applied extracellular [Na$^+$], following a period in NM, and the secretory response and change in [Na$^+$], is shown in Fig. 3. Nearly linear relationships were found for both the change in [Na$^+$], and the induction of AVP release, with the region of [Na$^+$], over which the changes occurred being closely correlated. Thus, the experiments provide additional evidence that sodium ions could act at an internal site on the exocytotic machinery to regulate or induce AVP release.

To investigate the possibility that Na$^+$ may have a direct action at an internal site and to eliminate changes in [Ca$^{2+}$], nerve endings were permeabilized to provide direct access of Na$^+$ and EGTA to the cytoplasm. The nerve endings were permeabilized with digitonin which collapses the membrane potential and allows direct control over the intracellular ionic concentrations [22]. After incubation in a medium containing primarily K$^+$-glutamate under Ca$^{2+}$-free (<10$^{-8}$ M) conditions (see Fig. 4 legend) Na$^+$ was substituted for K$^+$. The effect of replacement of K$^+$ by Na$^+$ is shown in Fig. 4. An AVP secretory response similar to that in the intact endings was observed (6.8 ± 0.7-fold increase above K$^+$-containing medium, n=12). These results do not exclude the possibility of Na$^+$ acting at an external site. However, they are consistent with previous reports showing enhanced secretion in the presence of Na$^+$ ionophores [12,23–25] or inhibitors of the Na$^+$ pump [10,11,26]. The above results thus support the hypothesis that Na$^+$ exerts its effect at an internal site.

Although often overlooked, a number of studies on neurotransmitter/neurohormone release have indicated that conditions which raise [Na$^+$], in nerve endings result in an increased release [6,9,10,27]. This was evidenced either by an increased frequency in spontaneous miniature excitatory post-synaptic potentials [10,26,28–30] or by potentiation of evoked responses [4,6,8,23,24]. Direct fusion of Na$^+$ containing liposomes at frog motor nerve terminals [31], use of Na$^+$-selective ionophores [12,23–25] and direct injection of Na$^+$ into the squid giant synapse [32] have also provided evidence for a regulatory role of internal Na$^+$ on secretion. A number of explanations for the effects of Na$^+$ have been put forward including: Na$^+$-induced Ca$^{2+}$ release from internal stores, increased Na$^+$-Ca$^{2+}$ exchange activity and alteration of the effectiveness of internal Ca$^{2+}$. Each of these involve Ca$^{2+}$ as the final mediator of secretion. Here, we report on the direct effect of Na$^+$ on release of neuropeptide from nerve endings incubated in a minimal medium. After studying in parallel experiments [Na$^+$], [Ca$^{2+}$], and AVP release our conclusion is that an increase of [Na$^+$], alone is sufficient to induce a robust and sustained increase of AVP release. Under similar experimental conditions, however, we have found considerable disparity between the change in [Ca$^{2+}$], and the secretory response. This suggests, possibly, that under these conditions Ca$^{2+}$ entry is diffusely spread over a nerve ending and, thus, local high Ca$^{2+}$ concentrations at release sites are not developed. It should be noted that the actual [Na$^+$], and [Ca$^{2+}$], at the sites of exocytosis may be considerably greater than those reported here which represent mean values for the nerve endings. However, the possibility of our results arising from the generation of Na$^+$, induced from locally high [Ca$^{2+}$], is unlikely, based on maintenance of secretory responsiveness in EGTA (Ca$^{2+}$-free) conditions in intact and in permeabilized nerve endings. Although the mechanism by which [Na$^+$] activates AVP release remains to be elucidated the present results strongly suggest that [Na$^+$], per se can modulate the release of neuropeptide.

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REFERENCES


Fig. 4. The effect of [Na$^+$] on the release of AVP from permeabilized nerve endings. Following permeabilization with digitonin the nerve terminals were perfused in a medium containing K$^+$ as the major monovalent cation. Na$^+$ (140 mM) was substituted for K$^+$ as indicated.

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