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Evidence for Release of Copper in the Brain: Depolarization-Induced Release of Newly Taken-Up ^{67}Copper

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ABSTRACT The potential importance of copper (Cu) in neurosecretion can be inferred from the demonstration that extracellular Cu modulates the secretory function of peptidergic neurons (in vitro studies) and from the presence of high Cu concentrations in nerve terminals and secretory vesicles, primarily within the soluble matrix of the latter. We have previously hypothesized that vesicular Cu is released from neurons undergoing exocytosis and that such extracellular Cu plays an important modulatory role in the central nervous system. To test this Cu release hypothesis, rat hypothalami were incubated under in vitro conditions for 1 or 2 hr with 20 nM radiolabeled Cu (^{67}Cu), and then ^{67}Cu release was stimulated by a depolarizing concentration (60 mM) of K^+. K^+ markedly ($P < 0.001$) stimulated ^{67}Cu release in a Ca^{2+}-dependent manner (stimulated release was 95 fmol/10 min/mg protein after 1 hr ^{67}Cu loading and 160 after 2 hr). These amounts of released ^{67}Cu account for about 10% of the total ^{67}Cu taken up by the tissue. These results indicate that part of the ^{67}Cu taken up by hypothalamic explants is directed into an intracellular compartment from where it can be released by a Ca^{2+}-dependent mechanism, thus providing strong support to our hypothesis that release of copper is operative in situ in the brain.

INTRODUCTION

It is well established that diverse substances can serve as triggers for exocytosis, the process by which secretory cells discharge their secretory substances. Several lines of evidence support the view that extracellular copper can serve as a trigger for exocytosis as well as a modulator of other triggers of exocytosis in the brain. One, copper acetate, administered systematically to female rabbits, leads to the release of the neuropeptide luteinizing, hormone-releasing hormone (LHRH) into the hypophysial portal blood and into the cerebrospinal fluid (Tsou et al., 1977; Pau and Spies, 1986). In a series of studies we have established that extracellular copper stimulates the release of LHRH from explants of the median eminence (the region of the mediobasal hypothalamus that is highly enriched with LHRH axonal terminals) and that one site of copper action in the LHRH secretory granule itself (Burrows and Barnea, 1982; Rice and Barnea, 1983; Barnea and Cho, 1984; Barnea and Colombani-Vidal, 1984; Colombani-Vidal and Barnea, 1986a,b,c). Others have shown that extracellular copper stimulates the release of luteinizing hormone, follicle-stimulating hormone, and adrenocorticotropic hormone from slices of bovine pituitary gland (LaBella et al., 1973). Two, copper has been shown to alter the binding properties of several types of plasma membrane receptors, e.g., acetylcholine (Gurwitz et al., 1984), adenosine (Marangos et al., 1983), opioid (Sadee et al., 1982), and LHRH receptors (Hazum, 1983). Three, we have shown that copper markedly amplifies the prostaglandin E_2 (PGE_2) stimulation of LHRH release from median eminence explants (Barnea et al., 1985, 1986a,b,c), and that this amplification is a post-PGE_2-receptor event (Barnea and Cho, 1987).

The question arises: What could be the source of extracellular copper? The blood is the sole source of tissue copper. Bearing in mind the existence of a blood-brain barrier to proteins and that more than 95% of circulating copper is complexed to proteins (Osterberg, 1980), it is hard to envision that the high levels of extracellular copper required for the manifestations of copper action could be derived from blood. We have previously proposed that the high extracellular levels of copper are generated within the brain itself, mainly by exocytotic release of copper along with other secretory substances (Barnea and Colombani-Vidal, 1984). The published reports that led to the formulation of this proposition are the following. The levels of brain copper are about one order of magnitude greater than those in blood (Donaldson et al., 1975; Merriam et al., 1979), suggestive of an active Cu uptake/accumulation process operative in the brain. Moreover, the secretory apparatus of neuronal

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tissue, i.e., the axonal terminals and secretory granules, is highly enriched with copper (Colburn and Maas, 1965; Rajan et al., 1976). Pertinent to this discussion is the presence of copper within the soluble matrix of secretory granules in association with copper-dependent enzymes such as dopamine-beta-hydroxylase (Phillips and Allison, 1977; Levine et al., 1983) and peptide alpha-amidating enzyme(s) (Eipper et al., 1983; Mains et al., 1984). The fact that the intravesicular soluble constituents of secretory granules are coreleased during exocytosis (Konings and DePotter, 1982; Levine et al., 1983; Mains et al, 1984) led us to hypothesize that copper is released from neuronal tissue during exocytosis. It is this release hypothesis that was tested in the present study.

MATERIALS AND METHODS

Animals

Male rats of the Long-Evans strain (9–10 weeks old, 190–220 g) were used. Rats were housed in temperature-controlled animal quarters under a daily lighting regimen of 12-hr light/dark cycle (on at 0600 hr) and had free access to lab chow and tap water.

In vitro incubation and measurement of ^{67}copper

Rats were decapitated, the hypothalamus was dissected out (the boundaries of the hypothalamic fragment extended from the anterior margin of the preoptic area to the posterior border of the mammilary bodies), and then incubated as previously described in detail (Barnea and Colombani-Vidal, 1984). Briefly, incubation was carried out in plastic chambers, constructed from 5-ml plastic disposable syringes, which were immersed in borosilicate glass vials containing 4 ml of Krebs-Ringer-phosphate (KRP) buffer, pH 7.4. Each chamber contained 6 to 7 coronal slices obtained from one-half hypothalamus divided longitudinally along the 3rd ventricle (about 0.7 mg tissue protein). Incubation was carried out under air at 37°C; after a 30-min period of equilibration; chambers were transferred to KRP containing ^{67}Cu complexed to histidine at a molar ratio of 1:2; and incubation was continued for the designated time. Incubation was terminated by rapidly rinsing (over 10–15 sec) each chamber in about 400 ml of 0.15 M NaCl kept at 37°C. Rinsed hypothalami were homogenized in 0.2 ml distilled water using a glass homogenizer and a plastic tube. Aliquots of the homogenate were taken for measurement of radioactivity using a Packard gamma counter at a setting of 165–220 keV. The cpm values were corrected for the 61-hr half-life decay of ^{67}Cu, and moles of released ^{67}Cu were computed from the specific activity of ^{67}Cu in the incubation medium. Protein was quantified in an aliquot of the homogenate by the method of Bradford (1976), using BSA (Sigma) as the reference standard.

The complex of ^{67}Cu with histidine (CuHis) was formed by mixing an aliquot of the commercial $^{67}CuCl_2$ (specific activity, 1.26×10^6 Ci/mol; Oak Ridge National Laboratories, Oak Ridge, TN) with L-histidine. HCl (Sigma Chemicals, St. Louis, MO) in KRP buffer to a final concentration of 20 nM copper and 200 nM histidine; the pH was adjusted to 7.4. The rationale for using a CuHis complex was based on our previous experience that complexed copper and not ionic is effective in modulating LHRH release from median eminence tissue under in vitro conditions (Barnea and Colombani-Vidal, 1984).

Efflux of ^{67}copper in vitro

Following loading the tissue with ^{67}Cu, hypothalami were rapidly rinsed and transferred to chambers containing ^{67}Cu-free KRP buffer for 5 successive 10-min periods. When K^+ stimulation was tested, the incubation medium of the 3rd successive period contained 60 mM K^+ ([NaCl] in the buffer was accordingly reduced to maintain osmolarity). An aliquot of each incubation buffer was taken for measurement of ^{67}Cu as described above.

Analysis of data

The significance of difference between group means was tested by Student's t-test or one-way analysis of variance. In the latter case, Duncan's test was employed for posthoc comparisons (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

It would have been desirable to test this copper release hypothesis by measuring the release of endogenous brain copper in response to an established stimulus of exocytosis such as depolarizing concentration of potassium (60 mM K^+). However, it is well documented that only a small fraction of the total cellular content of secretory substances is released during exocytosis. Therefore, even though the brain copper content is relatively high, the currently available procedures for quantification of copper in aqueous solutions do not possess the sensitivity required to reliably measure the minute amounts of endogenous copper released during exocytosis. Therefore, we have chosen the strategy of first labeling the releasable pool of Cu with radioisotope of high specific activity (^{67}Cu) and then stimulating the release of ^{67}Cu with 60 mM K^+.

First, we determined the time course of uptake of 20 nM ^{67}Cu by rat hypothalamic tissue incubated under in vitro conditions and found that the uptake proceeded linearly for 120 min (Fig. 1). To further assess the fate of this newly taken up ^{67}Cu, hypothalami were incubated with ^{67}Cu for 60 min and then with ^{67}Cu-free medium for 5 successive 10-min periods. It was found that the basal rate of ^{67}Cu efflux was biphasic: a high rate of efflux occurring during the first 10-min period

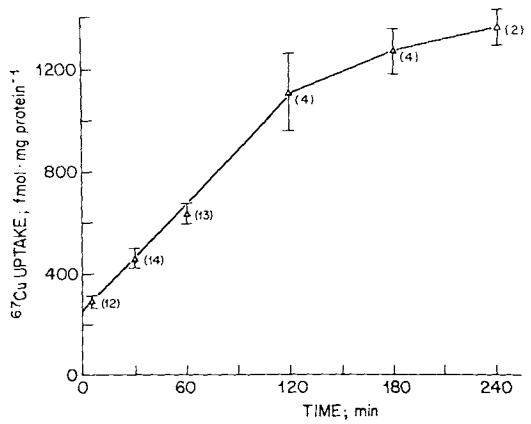

Fig. 1. Time course of ^{67}Cu uptake by hypothalamic explants. Hypothalamic explants were incubated with 20 nM ^{67}Cu-histidine (at a Cu:His molar ratio of 1:2) for 5–240 min. Each value is the mean ± SEM (N).

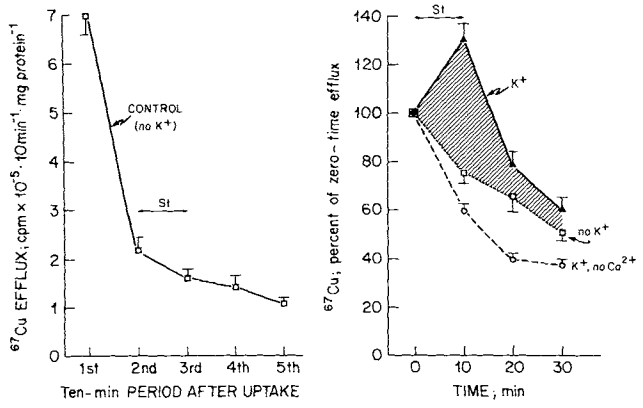

Fig. 2. Effect of 60 mM K^+ on ^{67}Cu efflux from hypothalamic explants. Hypothalamic explants were loaded for 60 min with ^{67}Cu as described in Figure 1, after which hypothalami were incubated in ^{67}Cu-free KRP for 5 successive 10-min periods. To assess the effect of depolarizing K^+, the incubation medium of the 3rd successive period (St) contained either regular KRP (no K^+), KRP containing 60 mM K^+ (K^+) or KRP containing 60 mM K^+ but no calcium (K^+, no Ca^{2+}). ^{67}Cu released into the medium was measured as described. Each value is the mean ± SE (N = 13–16). Left panel, time course of nonstimulated, basal ^{67}Cu efflux. Right panel, effect of 60 mM K^+ on ^{67}Cu efflux. For reference purposes, the prestimulation 10-min period of incubation was designated as zero time and ^{67}Cu efflux was taken as 100%; each time point thereafter is expressed as percent of zero-time efflux. The shaded area represents the net K^+-stimulated ^{67}Cu efflux. The statistical significance of the difference between group means (during St period) was evaluated using analysis of variance followed by Duncan's test; $P < 0.001$.

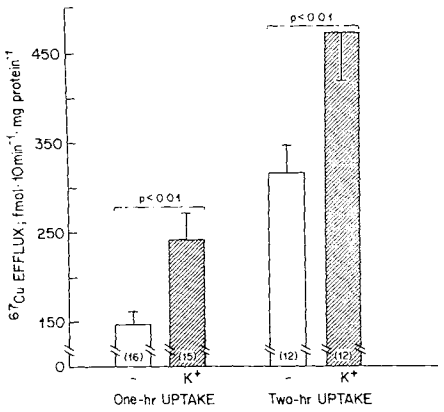

Fig. 3. K^+-stimulated ^{67}Cu efflux as a function of time of ^{67}Cu uptake. Hypothalami were incubated with ^{67}Cu for 60 or 120 min and then challenged with 60 mM K^+ as described in legends to Figures 1 and 2, respectively. Bars represent mean ^{67}Cu efflux from nonstimulated (open bars) and K^+-stimulated (hatched bars) hypothalami. The height of the bar indicates the mean ± SEM (N). K^+-stimulated efflux was found to be significantly ($P < 0.01$, t-test) greater than its respective control. Prestimulated ^{67}Cu efflux from the nonstimulated and stimulated groups of hypothalami was not significantly different, and it was 204.0 ± 23.0 and 189.0 ± 21.0, respectively, after 1-hr loading, and 518.0 ± 91.0 and 498.0 ± 55.0 fmol/10 min/mg protein, respectively, after 2-hr ^{67}Cu loading.

(most likely reflecting washout of the extracellular space and loosely bound ^{67}Cu) and a low rate of efflux progressively declining, occurring between the second and fifth successive periods of incubation (Fig. 2, left panel). Thus it appears that under nonstimulating conditions, efflux of ^{67}Cu has essentially stabilized by the second 10-min period of incubation in ^{67}Cu-free medium. Therefore, in the following experiments, the second period has been designated as the prestimulation period (zero time) and the ensuing third period as the stimulation (St) period.

Hypothalami were loaded with ^{67}Cu for 1 or 2 hr and then, during the stimulation period, hypothalami were exposed to either KRP buffer (no K^+), KRP buffer containing 60 mM K^+ (K^+) or KRP buffer containing 60 mM K^+ but no calcium (K^+, no Ca^{2+}). As shown in Figure 2, 60 mM K^+ markedly stimulated the release of ^{67}Cu into the medium. Taking the release during the prestimulation period as 100%, the release in the presence of 60 mM K^+ was 130%, whereas in the presence of control KRP buffer (no K^+), it was 75%, and in the presence of 60 mM K^+ and no Ca^{2+}, it was 60% of the prestimulated release (K^+ vs. no K^+, or K^+ vs. K^+, no Ca^{2+}; $P < 0.001$). Moreover, the net K^+-stimulated ^{67}Cu release was nearly twice as high after 2-hr loading with ^{67}Cu compared to 1-hr loading, and it was 160 and 95 fmol/10 min/mg protein, respectively (Fig. 3). It is noteworthy that these amounts of ^{67}Cu account for about 10% of the total newly taken up ^{67}Cu.

If these amounts of ^{67}Cu were released in situ in the brain, one can make a rough computation of the extracellular concentration of the released Cu using the equation:

$$\text{Released Cu} = \frac{\text{rate of Cu release/g brain}}{\text{rate of flow of interstitial fluid/g brain}}$$

Taking protein concentration in the brain = 10% of total wet weight, Cu release = 1.5 pmol/min g brain, and average flow rate of interstitial fluid = 0.1 μl/min/g brain (Szentistvanyi et al., 1984), then the extracellular concentration of exocytotically released Cu is 15 μM. This amount of Cu is most likely underestimated since it is based on the specific activity of the ^{67}Cu in the incubation medium. Nonetheless, 15 μM is within the range of Cu concentration required for the in vitro manifestations of the modulatory actions of copper in brain tissues, e.g. alterations of receptor properties (Sadee et al., 1982; Hazum, 1983; Marangos et al., 1983; Gurwitz et al., 1984) and peptide release (Burrows and Barnea, 1982; Rice and Barnea, 1983; Barnea and Cho, 1984; Barnea and Colombani-Vidal, 1984; Colombani-Vidal and Barnea, 1986a,b,c).

In summary, we demonstrate that depolarizing concentration of K^+ evokes the release of ^{67}Cu in a calcium-dependent manner, that only a small fraction of the newly taken up copper is released, and that the calculated potential extracellular concentration of the so released ^{67}Cu is within the range of copper concentration required for copper action. These findings strongly support our hypothesis that copper is released in situ in the brain and that such extracellular copper can serve as a neuromodulator. Although it is tempting to conclude that the copper is released by exocytosis, further investigation is required to establish the identity of the intracellular releasable pool of copper, the molecular form of the released copper, and the exact mechanism of copper release.

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