An investigation into the development of calcium-dependent neurotransmitter release from isolated growth cones

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The neuronal growth cone is a motile, expanded region at the tip of the growing axon or dendrite that is responsible for guiding neurites to their correct targets (Ramon y Cajal, 1890). This structure has received great attention in studies attempting to understand the mechanisms by which growing neuronal processes locate and recognize the appropriate sites with which they are to form synapses (Lockerbie, 1987). Once the correct target has been reached, the growth cone plays a central role in synaptogenesis, transforming into either the pre- or post-synaptic element (Rees, 1978). In this respect, neuronal growth cones are important structures in which to study the development of the mechanisms involved in neurotransmission.

It is now clear that in culture, neurotransmitters are present in growing neurites and that the growth cones of these neurites are able to release their neurotransmitters both spontaneously and in response to electrical stimulation (Hume et al., 1983; Young & Poo, 1983). In this laboratory, we have shown that growth cones isolated from developing rat forebrain are able to take up exogenous \(^{3}H\) GABA (Gordon-Weeks, 1987; Taylor et al., 1981). We have now attempted to correlate the \(^{3}H\) GABA release from subcellular fractions isolated from animals 5 days old in culture, where release was shown to resemble exactly that from the growth cones of Xenopus spinal neurones in culture, where release was shown to resemble exactly that present in the subcellular fractions isolated from older animals (Gordon-Weeks, 1987a). Therefore, although isolated growth cones display some means of \(^{3}H\) GABA release, the Ca\(^{2+}\)-dependent mechanism found in synaptosomes seems not to appear until some later point in development. This is in contrast to the electrically evoked release of GABA from growing neurites and that the growth cones of these neurites are able to release their neurotransmitters from subcellular fractions isolated from animals 5 days old or younger (Table I). After this age, a developmental increase in the magnitude of the release evoked from these fractions by high K\(^+\) was seen (Table I). This increased release was abolished by removal of Ca\(^{2+}\) from the superfusion medium, and was therefore Ca\(^{2+}\)-dependent. A much larger proportion of the total \(^{3}H\) GABA stores in synaptosomes was released in a Ca\(^{2+}\)-dependent manner in response to high K\(^+\) stimulation (Table I).

Electron microscopy

Subcellular fractions were prepared for electron microscopy essentially as described for synaptosomes (Gordon-Weeks et al., 1981) except that the sodium cacodylate buffer was 0.12 M (Gordon-Weeks, 1987a). Neuronal growth cones

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Table 1. \(K^+\) evoked release of \(^{3}H\)GABA from subcellular fractions isolated from neonatal rat forebrain and synaptosomes isolated from adult rat forebrain

P. Postnatal day. Results are expressed as the total elevated \(^{3}H\)GABA release (over baseline), following a 6 min, high K\(^+\) stimulus, as a percentage of \(^{3}H\)GABA present in the subcellular fractions at the beginning of the stimulation period. Values represent means \pm S.E.M. of at least five observations obtained in three separate experiments.

<table>
<thead>
<tr>
<th>Postnatal age</th>
<th>(0.2) mm-Ca(^{2+})</th>
<th>(2) mm-Ca(^{2+}) free (Ca(^{2+}) present)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1/P2</td>
<td>2.14 \pm 0.29</td>
<td>2.10 \pm 0.21</td>
</tr>
<tr>
<td>P4/P5</td>
<td>1.91 \pm 0.12</td>
<td>1.93 \pm 0.20</td>
</tr>
<tr>
<td>P7/P8</td>
<td>2.64 \pm 0.24</td>
<td>1.94 \pm 0.17</td>
</tr>
<tr>
<td>P10/P1</td>
<td>2.92 \pm 0.25</td>
<td>1.42 \pm 0.10</td>
</tr>
</tbody>
</table>

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Abbreviation used: GABA, \(\gamma\)-aminobutyric acid.
were recognized in subcellular fractions by previously described criteria (Gordon-Weeks & Lockerbie, 1984; Gordon-Weeks, 1987b; for discussion see Gordon-Weeks, 1988). Subcellular fractions isolated from rats 5 days old or younger, contained large numbers of growth cones and very few profiles containing synaptic vesicles. With increasing age, the numbers of growth cones present in the subcellular fractions declined and there was a concomitant increase in the numbers of particles containing synaptic vesicles, but no synapses, i.e., structures intermediate between growth cones and synaptosomes. Synaptosomes were rarely seen in these fractions.

The developmental increase in the number of synaptic-vesicle-containing profiles in subcellular fractions isolated from neonatal rat forebrain, correlated with the appearance of, and developmental increase in, the degree of Ca\(^{2+}\)-dependent, K\(^+-\)evoked \(^{3}H\)GABA release (Fig. 1). It appears from these results that GABA release from growth cones is initially independent of extracellular Ca\(^{2+}\), but that at a later point in development, once synaptic vesicles have appeared in the growth cone, depolarization of the growth cone results in the release of GABA by a Ca\(^{2+}\)-dependent mechanism. However, a much larger proportion of the total \(^{3}H\)GABA stores in synaptosome fractions is released in a Ca\(^{2+}\)-dependent manner in response to high K\(^+\) stimulation (Table 1). It is worth noting that even in subcellular fractions isolated from 10- and 11-day-old rats, those profiles containing synaptic vesicles contained fewer synaptic vesicles than synaptosomes isolated from adult rat forebrain.

**Fig. 1.** Graph showing the relationship between the percentage of synaptic-vesicle-containing profiles in subcellular fractions isolated from neonatal rat forebrain and the Ca\(^{2+}\)-dependency of K\(^+-\)-stimulated \(^{3}H\)GABA release from these fractions.

Ca\(^{2+}\)-dependent release was determined as the difference between the percentage of \(^{3}H\)GABA stores released in Ca\(^{2+}\)-containing Krebs' solution and that in Ca\(^{2+}\)-free Krebs' solution containing 10 mM-Mg\(^{2+}\), and is expressed as the mean percentage of at least five observations obtained in three separate experiments. The mean percentage of synaptic vesicle (S.V.)-containing particles was determined from six electron micrographs from each of two preparations at each age studied (see Table 1).

**Involvement of the plasma membrane GABA transporter in \(^{3}H\)GABA release**

A possible mechanism for \(^{3}H\)GABA release in response to high K\(^+\) stimulation in the absence of extracellular Ca\(^{2+}\) is reversal of the plasma membrane GABA transporter (Kanner & Radul, 1985). We have investigated this possibility by testing the effects of inhibitors of GABA transport on \(^{3}H\)GABA release from growth cone fractions isolated from the forebrains of 3-day-old rats and synaptosome fractions isolated from adult rat forebrain. Nipecotic acid, a well-known inhibitor of GABA transport into nerve terminals, potently inhibits GABA transport into isolated neuronal growth cones (Lockeibie et al., 1985). Nipecotic acid is transported into nerve terminals via the GABA transport mechanism and is able to store \(^{3}H\)GABA in synaptosomes isolated from adult rat forebrain. Involvement of the plasma membrane GABA transporter in \(^{3}H\)GABA release appears to be an important mechanism in K\(^+-\)-stimulated \(^{3}H\)GABA release.

The effect of SK&F 89976-A on uptake of \(^{3}H\)GABA into subcellular fractions isolated from neonatal rat forebrain and synaptosomes isolated from adult rat forebrain was determined. Subcellular fractions (80–100 µg of protein) were incubated for 12.5 min in the presence of \(^{3}H\)GABA (31 nM) and varying concentrations of SK&F 89976-A. Uptake was terminated by the addition of 10 ml of ice-cold Krebs' solution followed by rapid vacuum filtration, and filters were analysed for tritium content by liquid scintillation counting. Background accumulation, determined at 0–4°C, was less than 5% of control values. In experiments designed to test the effect of SK&F 89976-A on \(^{3}H\)GABA release, the compound was present throughout the superfusion.

We have found that while SK&F 89976-A appears to be acting on the same GABA transport system in growth cones and synaptosomes to inhibit uptake of \(^{3}H\)GABA (IC\(_{50}\) for inhibition of \(^{3}H\)GABA uptake into growth cones and synaptosomes was 1.3 × 10\(^{-6}\) M and 1.7 × 10\(^{-6}\) M, respectively), the drug is several orders of magnitude more effective in blocking K\(^+-\)stimulated \(^{3}H\)GABA release from growth cones than from synaptosomes (Fig. 2). SK&F 89976-A was found to block K\(^+-\)-stimulated \(^{3}H\)GABA release from growth cones at similar concentrations to those required to inhibit uptake (micromolar range). In contrast, much higher concentrations were required to inhibit the release from synaptosomes, suggesting that the effect on release from synaptosomes may not be specific. We are currently examining this possibility by testing the effects of the R and S enantiomers of SK&F 89976-A on \(^{3}H\)GABA release from growth cones and synaptosomes, as inhibition of GABA uptake into synaptosomes is reported to be stereoselective (Ali et al., 1985). Thus reversal of GABA transport appears to be an important mechanism in K\(^+-\)-stimulated \(^{3}H\)GABA release from growth cones in which a Ca\(^{2+}\)-dependent release mechanism has not yet developed, but does not appear to be an important mechanism in the K\(^+-\)-evoked release from synaptosomes, which is largely Ca\(^{2+}\)-dependent.

In summary, K\(^+-\)-stimulated \(^{3}H\)GABA release from isolated neuronal growth cones appears to be initially independ-
Fig. 2. Effect of SK&F 89976-A on $K^+$-evoked release of $[^3H]GABA$ from growth cones and synaptosomes

Growth cone fractions were isolated from 3-day-old rat forebrain (hatched bars) and synaptosomes were isolated from adult rat forebrain (open bars). Values represent $\pm$ S.E.M. of at least three separate experiments done in duplicate.

ent of extracellular $Ca^{2+}$ and supported largely by a mechanism involving reversal of the plasma membrane GABA transport system. As the growth cones become more mature, synaptic vesicles appear which correlates with the appearance of a $Ca^{2+}$-dependent release mechanism for $[^3H]GABA$.

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Solubilization of a glutamate-binding protein from brain membranes

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Introduction

Identification of specific binding sites for l-glutamate (Glu) in brain tissue has supported the hypothesis that this, or a closely related compound, serves as the major excitatory neurotransmitter in many species. Specific binding to neuronal membranes was independently first observed by Michaelis (Michaelis et al., 1974) and Roberts (Roberts, 1974). Reports of solubilized Glu-binding sites appeared shortly thereafter (Michaelis, 1975). Solubilization of the binding sites from neuronal membranes was accomplished using Triton X-100 and exposure to pH 9.5 (which increases the efficiency of this detergent in dissolving lipid bilayers). Furthermore, this solubilized activity could be purified to homogeneity using batch affinity adsorption (on l-glutamate-lectinated bovine serum albumin/glass fibre, (GBP)) followed by lectin chromatography (on concanavalin A-linked Sepharose). Since this time, excitatory amino acid receptors have been divided into three major subtypes dependent on their selective sensitivity to the agonists N-methyl-D-aspartate, quisqualate and kainate (Watkins & Evans, 1981; McLennan, 1981); however, these compounds fail to displace the binding of $[^3H]Glu$ to this solubilized binding site.

Different detergents used to solubilize ligand-binding sites from nerve terminals can have different effects on the pharmacology of the binding sites (Wouters et al., 1981; Demoliou-Mason & Barnard, 1984), thus it was of interest to attempt to isolate this Glu-binding protein (GBP) using a different detergent. Other conditions used in the original isolation scheme for this GBP which might influence the pharmacology of the protein were its exposure to high pH (pH 9.5, for solubilization) and high salt (1 M-KCl, for elution from the GBP). It was therefore of interest to attempt isolation of this GBP avoiding the conditions of high salt and high pH. An alternative isolation scheme was proposed involving solubilization with Triton X-100 or potassium cholate at pH 7.5, followed by affinity adsorption and elution of bound proteins from the GGF with ligand (0.1 mM-l-Glu).

Materials and methods

All chemicals used were of analytical grade. Lectins were purchased from Vector Laboratories, Burlingame, CA, U.S.A., and $[^3H]$-glutamate (39 Ci/mmol) was supplied by Amersham International, U.K.

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