Regulated Plasmalemmal Expansion in Nerve Growth Cones

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Abstract. To study the mechanisms underlying plasmalemmal expansion in the nerve growth cone, a cell-free assay was developed to quantify membrane addition, using ligand binding and sealed growth cone particles isolated by subcellular fractionation from fetal rat brain. Exposed versus total binding sites of 125I-wheat germ agglutinin were measured in the absence or presence of saponin, respectively, after incubation with various agents. Ca^{2+}-ionophore A23187 in the presence of Ca^{2+} increases the number of binding sites (B_{max}) but does not change their affinity (K_{D}), indicating that new receptors appear on the plasma membrane. Similarly, membrane depolarization by high K+ or veratridine significantly induces, in a Ca^{2+}-dependent manner, the externalization of lectin binding sites from an internal pool. Morphometric analysis of isolated growth cones indicates that A23187 and high K+ treatment cause a significant reduction in a specific cytoplasmic membrane compartment, thus confirming the lectin labeling results and identifying the plasmalemmal precursor. The isolated growth cones take up γ-amino butyric acid and serotonin, but show no evidence for Ca^{2+}-dependent transmitter release so that transmitter exocytosis is dissociated from plasmalemmal expansion. The data demonstrate that plasmalemmal expansion in the growth cone is a regulated process and identify an internal pool of precursor membrane.

The neuronal perikaryon is the source and site of synthesis of macromolecular components necessary for neurite growth. Expansion of the neurite's plasmalemma, however, occurs primarily at the growth cone. Labeling studies with fluorescent lectins or carmine particles (Bray, 1970; Feldman et al., 1981), pulse-chase experiments with ferritin-labeled lectins and with phospholipid precursors (Pfenninger and Maylie-Pfenninger, 1981; Pfenninger and Johnson, 1983), and autoradiography of radiolabeled membrane proteins (Tessler et al., 1980; Griffin et al., 1981) support this concept. There is evidence that new membrane components, proteins and lipids, are inserted into the growth cone plasmalemma in preassembled form. The plasmalemmal precursor has been suggested to form the large clear vesicles characteristic of growth cones (Pfenninger and Bunge, 1974; Pfenninger and Maylie-Pfenninger, 1981; Cheng and Reese, 1987; Shea and Sapirstein, 1988) and to be delivered from the perikaryon by rapid axonal transport (Pfenninger and Johnson, 1983). A recent study has shown that anterogradely transported vesicles are unloaded from microtubules in the proximal region of the growth cone (Cheng and Reese, 1987). For studying plasmalemmal expansion, the use of isolated nerve growth cone particles, sheared-off and resealed growth cone fragments (GCPs); Pfenninger et al., 1983), has the great advantage of separating cellular sites of synthesis from those of insertion. Furthermore, GCPs contain cytoplasmic membrane compartments that are likely to include the plasmalemmal precursor. To analyze the mechanism of insertion, we use an in vitro assay involving measurement of superficially exposed vs total ligand binding sites in GCPs. The ligand of choice is wheat germ agglutinin (WGA) because its binding sites are abundant and easily detectable in growth cones (e.g., Pfenninger and Maylie-Pfenninger, 1981; Greenberger and Pfenninger, 1986). These ligand binding assays are correlated with morphometric analyses and show Ca^{2+}-regulated externalization of membrane. Some of these data have been presented previously in abstract form (Lockerbie, R., J. Bower, and K. Pfenninger. 1989. J. Cell Biol. 109:229a).

Materials and Methods

Materials

Hepes, N-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), HRP, N-acetyl-L-glucosamine (GlcNAc), luciferin-luciferase reagent, ATP, NADH, pyruvate, veratridine, BSA, EGTA, saponin, peptatin, PMSF, leupeptin, amino-oxyacetic acid (AOAA), diaminobutyric acid (DABA), imipramine (IMP), and desmethyl imipramine (DMI) were all from Sigma Chemical Co. (St. Louis, MO). Aprotinin (Trasylool) was from Bayer Leverkusen, W. Germany (distributed by FBA Pharmaceuticals, West Haven, CT). A23187 was from CalbiochemBehring Corp. (La Jolla, CA) and Fura-2 AM ester was from Molecular Probes Inc. (Junction City, OR). Benzotropine was from Merck, Sharp and Dohme (St. Louis, MO). Na^{125}I (30 mCi/mmol) was from ICN K & K Laboratories Inc. (Plainview, NY) and [3H]-GABA (70 Ci/mmol) from Amersham Corp. (Arlington Heights, IL). [3H]-Serotonin (5-HT) (20.2 Ci/mmol), [3H]-carboxy insulin (2.5 mCi/g), and [3H]-H2O (1 mCi/ml) were from NEN (Boston, MA).

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1. Abbreviations used in this paper: DABA, diaminobutyric acid; DMI, desmethyl imipramine; GABA, γ-amino butyric acid; GCP, growth cone particle; 5-HT, serotonin; IMP, imipramine; LDH, lactate dehydrogenase; SK, sucrose-Krebs buffer; WGA, wheat germ agglutinin.
Preparation of GCPs

Modifications of the methods described by Pfenninger et al. (1983) and Hyman and Pfenninger (1985) were used to prepare sealed GCPs from day 18 fetal rat brains. The GCP fraction obtained from the discontinuous sucrose gradient was diluted by the dropwise addition of 6-7 vol of cold 0.32 M sucrose with 1 mM TES buffer (pH 7.3), 1 mM MgCl₂, and 100 Kallikrein inactivator U/ml (KI/ml) aprotinin. The diluted GCP fraction was layered on top of a cushion of MaxiSolv oil and spun at 4000 gmax for 60 min at 4°C in an SW28 rotor (Beckman Instruments, Inc., Palo Alto, CA). The interface between the load and cushion was resuspended gently in a modified Krebs medium (Fried and Blaustein, 1978) of the following composition (in mM): sucrose, 180; NaCl, 50; KCl, 5; HEPES, 22; glucose, fetal rat brains. The GCP fraction obtained from the discontinuous sucrose will be referred to as

Biochemical Assays of GCP Integrity

times at 37°C before spinning at 15,000 gmax for 5 rain. The pellet was

calcium-free (Ca²⁺-free SK buffer), Na⁺-free (Na⁺-free SK buffer), and/or

equal volume of 0.8 M KOH and spinning down the insoluble KC10₄ salt

ATP Assay. GCPs (100 μg protein) were incubated in SK buffer for vari-
utations and pellets were counted in Eppendorf tubes precoated with SK buffer containing 0.5% BSA. Appro-

Quanti1ative EM. GCPs (100 μg protein) were incubated at 37°C for 10 min in the presence or absence of 15 mM NaF, 0.5 mM DNP, and 3 mM NaH₂PO₄ before spinning at 15,000 gmax for 5 min. The pellet was extracted for 30 min at 4°C in 0.8 M HC10₄ before neutralizing with an equal volume of 0.8 M K OH and spinning down the insoluble KClO₄ salt at 10,000 gmax (10 min at 4°C). The supernatant was then assayed for ATP by the luciferin-luciferase method essentially as described by Wiener et al. (1974). Upon addition of ATP standard (0-160 pmol ATP) or ATP-containing sample to the assay mixture, the vial was shaken, immediately placed in a Packard Tri-carb liquid scintillation counter (coincidence switch Sfe/Sb2 according to Daniell et al. (1987). In addition to correction for autofluorescence (~35% of total fluorescence), a correction for leak of emission intensities at 350 (F350) and 380 nm (F380) excitation. A cali-

Binding Assay. Unless stated otherwise, GCPs were incubated in SK buffer for 10 min at 37°C before the single or combined addition of agents for various time periods (see Results). Samples were then placed on ice for ~60 s before the binding assay was performed. Binding assays were carried out in Eppendorf tubes precoated with SK buffer containing 0.5% BSA. Appro-

Quantitative EM. GCPs (100 μg protein) were incubated at 37°C for 10 min in the presence or absence of A23187 (10 μM) or for 5 min with high K⁺ SK buffer before being fixed, pelleted, and processed for EM as described previously (Pfenninger et al., 1983). For each condition (control, A23187, high K⁺), thin sections were cut at approximately equivalent levels throughout each pellet, and a total of 100 micrographs were taken randomly at a primary magnification of 21,000 using a Phillips CM10 electron microscope. The micrographs were enlarged ~2.4 times, given random code numbers and then analyzed (double-blind test). For stereologic analy-

Endocytosis Studies

Hrp was iodinated by the Chloramine-T method (Hunter and Greenwood, 1962), and free iodine was removed by exhaustive dialysis against PBS. The ¹²⁵I-HRP was electrophoretically pure, and specific activity was ~0.2 mCi/mg protein. GCPs (50 μg protein) were preincubated for 10 min at 37°C or at 4°C before being added to a final volume of 200 μl SK buffer containing 0.1% BSA and ~0.3 μg/ml [¹²⁵I]-GABA, with or without 0.005% (w/t) saponin. The assay mix was then layered onto 0.3 ml of SK buffer containing 0.5% BSA in an Eppendorf tube and centrifuged for 60 s at 15,000 gmax. Radioactivity bound to the pellet (total binding) was estimated using a 5500 gamma counter (Beckman Instruments, Inc.). All assays were performed in triplicate and included blanks in which the GCPs were omitted from the incubation mix. Nonspecific binding was determined by adding 0.2 M GlcNAc to the incubation mix and subtracted from total binding to calculate specific binding. For analyzing saturation kinetics, GCPs were incubated as described above with increasing concentrations (0.8-100 μg/ml) of unlabeled WGA and 0.3 μg/ml [¹²⁵I]-WGA.

Transmitter Studies

GABA Uptake. Unless stated otherwise, GCPs (50-100 μg protein) were preincubated for 10 min at 37°C in SK buffer containing 100 μM AOA to inhibit γ-aminobutyric acid (GABA)-transaminase (Gardner and Ritchie, 1981). They were then preincubated for 30 min in a final volume of 1 ml SK buffer containing 14 nM [³H]-GABA (70 Ci/mmol) diluted with unlabeled GABA to a final concentration of 5 μM (Gordon-Weeks et al., 1984; Lockerbie et al., 1985).

Serotonin Uptake. Unless stated otherwise, GCPs (50-100 μg protein) were preincubated for 10 min at 37°C in SK buffer containing 30 μM pargy-
Results

Integrity of Isolated Growth Cones (GCPs)

Control and experimental SK buffers are intact, bounded by a microscopic examination reveals that GCPs incubated in compound without transmitter, followed by rapid vacuum filtration on GF/F filters (Whatman Inc., Clifton, NJ). Filters were washed once with cold buffer and counted using Filtron-X (National Diagnostics Inc., Summerville, NJ) as scintillator. All assays were performed in triplicate and included blanks in which the GCPs were omitted from the incubation mix.

Release. GCPs were preincubated with [3H]-GABA or [3H]-5-HT as described above and placed onto GF/F filters (Whatman Inc.) presoaked in Ca2+-free SK buffer (~200 μg protein/2.5-cm filter for [3H]-GABA release; ~500 μg protein/filter for [3H]-5-HT release). The filters were held in superfusion chambers as described by Bowyer et al. (1987) and treated as follows (Locke et al., 1985). GCPs were washed for 14 min with SK buffer at a flow rate of 0.5 ml min⁻¹. Thereafter, 2-min fractions were collected directly into scintillation vials. Superfusion was continued for 6 min with SK buffer to establish a baseline rate of release. The medium was then changed to high-K⁺ SK buffer or 10 μM A23187 in SK, and superfusion continued for another 10 min before changing back to SK buffer and superfusion for a final 10 min. Where appropriate, CaCl₂ was omitted from the SK buffer. After superfusion, the filters with the remaining radioactivity and the fractions were counted. The amount of tritium released was used to calculate the fractional release rate, i.e., the radioactivity released in each 2-min fraction expressed as a proportion (percentage) of the radioactivity present in the GCPs at the time the fraction was collected.

Other Methods

Preparation of Synaptosomes. Synaptosomes were prepared from grey matter of adult rat brain essentially according to Cohen et al. (1977) with modifications described by Hyman and Pennington (1985). The interface between the 1.0/1.2 M sucrose solutions was collected and diluted in 10 vol of 0.32 M sucrose containing 1 mM TES, 1 mM MgCl₂, and 100 KIU/ml aprotinin. The diluted synaptosomes were then processed as described for the preparation of GCPs.

Protein Measurement. Protein was measured by the method of Bradford (1976) with the modifications suggested by Friedenauer and Berlet (1989), using BSA as the standard.

Results

Integrity of Isolated Growth Cones (GCPs)

The membrane addition assay developed for GCPs is critically dependent on them being intact and viable. The integrity of GCPs was assessed ultrastructurally and by measuring the uptake of membrane-impermeant molecules as well as the release of LDH, a cytosolic marker. Electron microscopic examination reveals that GCPs incubated in control and experimental SK buffers are intact, bounded by a continuous plasma membrane (Fig. 1). That GCPs are sealed is supported biochemically by their exclusion of 32P-ATP (uptake, measured as 32P incorporation into protein, requires the presence of saponin; data not shown) and of insulin (see below). Furthermore, the extracellular tracer HRP is not taken up under various incubation conditions as shown further below. Fig. 2 A illustrates that >80% of LDH activity is associated with pelleted GCPs at the onset of incubation and that uptake does not measurably leak from GCPs during a 30-min treatment at 37°C with either nondepolarizing SK or depolarizing high-K⁺ SK buffer. Only after 60 min of incubation does one start to see a marked increase of LDH in the GCP supernatant. To assess viability of GCPs, ATP production was measured. GCPs taken from the cold sucrose gradient contain only 0.6 nmol ATP/mg protein. However, after 10 min incubation at 4°F or 37°C in SK buffer, ATP increases to 6 nmol/mg protein and remains close to that level for at least 30 min (Fig. 2 B). The ATP content inside GCPs, their volume was measured. Based on 3H₂O distribution and 125I-inulin exclusion as described, the average GCP volume is estimated to be 10.2 μl/mg protein (mean of two determinations, 10.05 μl/mg and 10.31 μl/mg). Therefore, GCPs raise their ATP concentration to ~0.6 mM initially and maintain >0.4 mM ATP for up to 60 min at 37°C. ATP production is blocked by >95% in the combined presence of DNPI and NaN₃, inhibitors of oxidative phosphorylation, with or without NaF, a glycolysis inhibitor (Marden et al., 1982) (Fig. 2 B). Residual ATP is as low as the background, i.e., the level found after incubating GCPs for 240 min at 37°C in the presence of the inhibitors and then boiling them in acid for 10 min.

Intracellular Ca²⁺ levels are a further, important parameter of GCP integrity and viability, and proper Ca²⁺ regulation is critical for the experiments described here. Fig. 3 A shows the values of free Ca²⁺ in GCPs measured with the fluorescent probe FURA-2. Resting Ca²⁺, initially ~100 nM, is dependent on both incubation time at 37°C and extracellular Ca²⁺. In Ca²⁺-containing medium, cytosolic Ca²⁺ increases to ~200 mM during a 30-min control incubation. Fig. 3, B and C show that the addition of veratridine (100 μM in SK buffer) or KC1 (high-K⁺ SK buffer) increases free Ca²⁺ in GCPs within 5 min by 55 and 93%, respectively, relative to untreated controls (compare Fig. 3 A). These increases are completely blocked in Ca²⁺-free SK buffer. A nonfluorescent derivative of A23187, 4-bromo A23187 (Molecular Probes Inc.) was found to severely quench FURA-2 fluorescence under our conditions, precluding analysis of its effect on resting Ca²⁺. We conclude that GCPs maintain a physiological Ca²⁺ gradient across their plasmalemma, and membrane depolarization triggers Ca²⁺ influx as in cultured growth cones (Mattson and Kater, 1987).

Externalization of WGA Sites

To quantify membrane addition in GCPs, superficially exposed vs total membrane glycoconjugates are labeled with [125I]-WGA in the absence or presence of low concentrations of saponin, respectively. The difference between the two measurements represents internal WGA sites. These measurements are made after pretreatment of the GCPs with various agents to investigate the mechanisms underlying mobilization of the internal WGA sites to the plasmalemma.

[125I]-WGA labeling is carried out using 5-100 μg GCP protein, and binding is linear in this range, reaching an equilibrium within ~15 min. Nonspecific binding is <20%. WGA binding generates an approximately linear Scatchard plot and saturates at a concentration below 10 μg/ml (~3 x 10⁻⁷ M). However, multiple WGA receptors are known to be present on GCPs (Greenberger and Penningter, 1986) so that linear regressions and "apparent dissociation constants" presented here are valid operationally only, for comparisons between the experiments, and not for the definition of a single binding site. The Scatchard plots in Fig. 4 are from a representative experiment and show that saponin (0.005% wt/vol) increases the number of binding sites (Bmax) about twofold without significant change in their affinity (apparent K₀ = 40 and 64 nM). Similar increases in specific binding...
Figure 1. EM of GCPs spun onto Maxidens oil and resuspended in experimental and control SK buffers. (A and D) Control SK; (B) SK containing $10^{-5}$ M A23187; (C) high-K$^+$ SK. Open arrows point at lysed GCP “ghosts.” Note, however, that most GCPs are intact and surrounded by continuous plasma membrane. Asterisks mark large, vacuole-like structures that may be generated, in part, by sectioning through concavities (triangles). Arrowheads point at clusters of vesicles (<180 nm) characteristic of growth cones in culture and in vivo. Magnifications, (A–C) ×11,600; (D) ×25,500. Bars, 1 μm.
can be measured after lysis of GCPs in hypotonic medium followed by repetitive freeze-thaw cycles or with saponin concentrations from 0.0025 to 0.05% wt/vol (data not shown).

**Ca²⁺- and Depolarization-dependent Externalization of WGA Sites**

Externalization of WGA sites in GCPs is shown in Table I. Incubation of GCPs for 10 min at 37°C with 10 μM A23187 in SK buffer, or for 5 min with high K⁺ SK buffer, increases surface labeling significantly from 36 to 52%, by a factor of >1.4. However, the number of total sites remains constant regardless of the treatment. Considering that GCPs remain sealed during incubation, this suggests mobilization of internal sites to the surface. The ionophore effect is concentration-dependent, detectable at 10⁻⁸ M, and reaching a plateau at 10⁻⁶ M with 1.2 mM Ca²⁺. A23187 (10⁻³ M) in the presence of 1.2 mM Ca²⁺ increases the ratio of exposed

**Graphs and Figures**

Figure 2. (A) Lactate dehydrogenase assays on GCPs. GCPs were incubated for various times at 37°C in control SK or high-K⁺ SK buffer. The samples were then centrifuged and the pellets and supernatants assayed for LDH activity. Total LDH activity was 0.027 ± 0.002 μmole NADH oxidized min⁻¹ mg⁻¹ protein. Shown are the means ± SEMs of four experiments. (B) ATP production in GCPs. GCPs were incubated for various times at 37°C in the presence or absence of metabolic inhibitors before measuring ATP levels by the luciferin-luciferase assay. Shown are the means ± SEMs of three experiments.

Figure 3. FURA-2 measurements of free Ca²⁺ levels in GCPs. (A) GCPs preloaded with FURA-2 were incubated for various times at 37°C in SK buffer or in Ca²⁺-free SK buffer. At the times indicated fluorescence was measured to determine intracellular free Ca²⁺ levels. Shown are the means ± SEMs of four or more experiments with GCPs in SK buffer and the means ± SEMs of two experiments with GCPs in Ca²⁺-free SK buffer. Note the time-dependent increase in cytoplasmic Ca²⁺ in SK buffer. (B and C) GCPs preloaded with FURA-2 were incubated for 10 min at 37°C in SK buffer or Ca²⁺-free SK buffer before the addition of 100 μM veratridine (B) or high-K⁺ SK (C). Fluorescence measurements were then made at the times indicated to determine intracellular free Ca²⁺ levels. Shown are the changes in Ca²⁺ levels induced by membrane depolarization relative to the untreated controls shown in A (each point represents the mean ± SEM of four experiments). Veratridine causes a gradual increase in intracellular Ca²⁺ (significance of increase between first and last measurement, P < 0.001), which is dependent upon the presence of extracellular Ca²⁺. High-K⁺ treatment results in a much more rapid increase in intracellular Ca²⁺ so that, relative to control (Ca²⁺-free SK buffer), the first measurements are already significantly different (P < 0.001). Due to data scatter, the further increase in Ca²⁺, between 1 and 5 min of incubation, is only marginally significant (P < 0.1).
over total binding sites gradually; a maximum is reached after 10 min incubation (Fig. 5 A). (The control ratio remains low; for a 10-min control, see Table I.) Externalization of WGA binding sites is similar in amplitude, but much more rapid in high-K⁺ SK compared to A23187 in SK; the maximal effect of high K⁺ is seen by 2-5 min incubation (Fig. 5 B). Ca²⁺ dependence of the effects of A23187 and high K⁺ is shown in Fig. 6 A: the two treatments performed in Ca²⁺-free SK buffer completely block the externalization of WGA binding sites. In additional experiments, veratridine, a drug that opens Na⁺ channels, was tested for its effects on WGA receptor externalization. Fig. 6 B shows the large increase in exposed WGA sites caused by veratridine in control SK buffer and the inhibition of this effect by deletion from the buffer of either Na⁺ or Ca²⁺.

Additional WGA binding studies were performed on non-permeabilized GCPs incubated in control SK or with A23187 in order to ascertain that there was no change in affinity of the exposed receptors. The Scatchard analysis of a representative experiment is shown in Fig. 7. The averaged apparent KD values for control and A23187-treated GCPs, from three experiments, remain constant at 51 ± 3 nM (mean ± SEM). However, A23187 increases Bmax from 68 to 105 nmol/mg GCP protein, by 55 ± 4% (mean ± SEM, n = 3), indicating the mobilization of glycoconjugates to the cell surface. Whether this process requires energy was assessed with the metabolic poisons DNP and NaN₃, which reduce ATP in GCPs by >95% to background level (Fig. 3 B). Incubation of GCPs with these poisons before (10 min) and during A23187 treatment has no effect on the externalization of WGA sites (data not shown).

Quantitative EM

Quantitative EM was undertaken to support the results obtained by WGA labeling, and to identify the changing membrane compartment, the plasmalemmal precursor, in GCPs. As shown in Fig. 1, GCPs incubated in SK are largely intact even after treatment with A23187 or high K⁺. However, some of them appear lysed, and large vacuole-like structures are more common than in GCPs fixed immediately after gradient fractionation (see Pfenninger et al., 1983). Some of these vacuolar structures are in reality non-internal membrane compartments and result, in part, from the cross-sectioning of plasmalemmal invaginations. This was demonstrated by incubating GCPs removed from the density gradient in ferritin (22 mg/ml) for 5 min on ice and then fixing them in the presence of the tracer (Fig. 8). Therefore, the morphometric analysis was restricted to clearly identifiable, unlysed GCPs, and the internal vesicular structures were separated into two classes on the basis of size (smaller or larger than 180 nm diameter) to discriminate, at least roughly, between vesicles and the vacuole-like structures. Table II shows the effects of A23187 and high K⁺ treatment on GCPs.

Table I. Externalization of WGA Binding Sites in GCPs

| Treatment     | Total: es + is | Surface: es | Internal: is |
|---------------|----------------|-------------|--------------|
|               | % of total     | es / es + is x 100% | % of total |
| Control       | 46,300 ± 4100  | 16,900 ± 1500 | 29,400       |
|               | 100%           | 36          | 64%          |
| A23187 (10⁻⁴ M) | 44,900 ± 4100  | 23,500 ± 2200 | 21,400       |
|               | 100%           | 52          | 48%          |
| High K⁺ (25 mM) | 42,100 ± 2700  | 22,100 ± 2500 | 20,000       |
|               | 100%           | 52          | 48%          |

GCPs were incubated for 10 min at 37°C in SK buffer with or without A23187, or for 5 min in high-K⁺ SK buffer. Then they were labeled for 15 min at 4°C with [¹²⁵I]-WGA (0.3 μg/ml) in the presence or absence of saponin. Shown are the means ± SEM of eight or more measurements for each experiment. (es) Exposed binding sites; (is) internal binding sites.

* These values are raw, not normalized; for a given experiment, total binding sites were the same after all three incubations. Single analysis of variance gives significant differences between treatments and control: $ p < 0.05$; $ $ p < 0.01.

Figure 4. Scatchard plots of [¹²⁵I]-WGA binding to GCPs in the presence or absence of saponin. GCPs were incubated for 15 min at 4°C with [¹²⁵I]-WGA (0.3 μg/ml) diluted with unlabeled WGA over a range of concentrations (0.8-10.0 μg/ml). Each point is the mean of triplicate determinations from a single representative experiment. Linear regressions and binding constants are used operationally only (see text). A two-site fit would describe the "superficial" points equally well, but the linear regression shown (r² = 0.75) generates KD and Bmax values that are essentially identical to those resulting from another experiment of the same type, seen in Fig. 7 (control; for linear regression, r² = 0.95). r² for the linear regression of "total" points equals 0.83. Note that KD values are essentially the same for intact and permeabilized GCPs.
Exposed vs total WGA binding sites in A23187- and high-K⁺-treated GCPs. (A) Time course of A23187 stimulation. GCPs (~50 μg protein) were incubated in SK buffer for various times at 37°C in the presence or absence of 10 μM A23187. Specific ¹²⁵I-WGA binding was then measured in the presence or absence of saponin to determine the number of total vs exposed binding sites, respectively. Shown are the means ± SEMs of three experiments. Note that control binding ratios do not change during incubation. For comparison with a control at 10 min, see Table I. (B) Time course of K⁺ stimulation. GCPs (~50 μg protein) were incubated for various times at 37°C in control or high-K⁺ SK buffer before measuring specific ¹²⁵I-WGA binding in the presence or absence of saponin. Shown are the means ± SEMs of three experiments. The decline in binding ratios observed at longer incubation times in both experiments may be due to GCP aggregation which would affect binding to intact but not to saponin-treated GCPs.

Figure 5. Exposed vs total WGA binding sites in A23187- and high-K⁺-treated GCPs. (A) Time course of A23187 stimulation. GCPs (~50 μg protein) were incubated in SK buffer for various times at 37°C in the presence or absence of 10 μM A23187. Specific ¹²⁵I-WGA binding was then measured in the presence or absence of saponin to determine the number of total vs exposed binding sites, respectively. Shown are the means ± SEMs of three experiments. Note that control binding ratios do not change during incubation. For comparison with a control at 10 min, see Table I. (B) Time course of K⁺ stimulation. GCPs (~50 μg protein) were incubated for various times at 37°C in control or high-K⁺ SK buffer before measuring specific ¹²⁵I-WGA binding in the presence or absence of saponin. Shown are the means ± SEMs of three experiments. The decline in binding ratios observed at longer incubation times in both experiments may be due to GCP aggregation which would affect binding to intact but not to saponin-treated GCPs.

Figure 6. Ca²⁺ dependence of the effect of membrane depolarization on WGA binding. (A) A23187 and K⁺ stimulation. GCPs (~50 μg protein) were incubated for 10 min at 37°C in the presence or absence of A23187 (10 μM) in SK buffer, or for 5 min in high-K⁺ SK buffer in the presence or absence of extracellular Ca²⁺. Specific ¹²⁵I-WGA binding was then measured in the presence or absence of saponin. Shown are the means ± SEMs of three experiments. (B) Veratridine stimulation. GCPs (~50 μg protein) were incubated for 10 min at 37°C in SK buffer with or without 100 μM veratridine, in the presence or absence of Ca²⁺ (Ca²⁺-free SK) or Na⁺ (Na⁺-free SK). Specific ¹²⁵I-WGA binding was then measured in the presence or absence of saponin. Shown are the means ± SEMs of two experiments.

In influx and membrane depolarization. The >180-nm vesicles or vacuoles, which constitute ~20% of total GCP membrane, do not seem to undergo changes in their relative membrane area in either A23187- or high-K⁺-treated GCPs, compared to control. Mitochondrial volumes and surface
Figure 7. Scatchard analysis of externalized WGA binding sites in GCPs. GCPs were incubated for 10 min at 37°C with or without 10 μM A23187. Kinetics of WGA binding to exposed glycoconjugates were then measured by labeling GCPs (in the absence of detergent) for 15 min at 4°C with [125I]-WGA (0.3 μg/ml) diluted with unlabeled WGA over a range of concentrations (0.8-10.0 μg/ml). Each point is the mean of triplicate determinations from a single representative experiment. Linear regressions, r² = 0.95 and 0.99 for control and A23187, respectively.

Discussion

Plasmalemmal expansion and turnover of plasmalemmal components are believed to be the result of fusion of Golgi-derived vesicles with the cell surface (for review see, e.g., Burgess and Kelly, 1987). In the various nonneuronal systems investigated, plasmalemmal insertion of membrane components appears to be a constitutive process, and accumulations of plasmalemmal precursor vesicles have not been observed (Burgess and Kelly, 1987). During neurite growth, plasmalemmal expansion occurs at a very rapid rate (~0.5 μm²/min per mammalian neurite; Pfenninger and Maylie-Pfenninger, 1981), appears to be focused to the growth cone (Bray, 1970; Feldman et al., 1981; Griffin et al., 1981; Pfenninger and Maylie-Pfenninger, 1981), and must be tightly correlated with neurite growth and advancement. Of further interest is the presence in growth cones of clusters of vesicles which could be plasmalemmal precursors (Pfenninger and Bunge, 1974; Pfenninger and Maylie-Pfenninger, 1981; Cheng and Reese, 1987; Shea and Sapirstein, 1988) and, thus, may suggest a regulated membrane insertion phenomenon.

It is of general interest, therefore, to analyze the mecha-
Figure 8. Ferritin distribution after incubation of GCPs for 5 min on ice with 22 mg/ml of the tracer. Incubated GCPs were immediately fixed and processed for ultrastructural analysis as described. Small arrows point at ferritin detected in large, vacuolar structures, indicating that they are invaginations of extracellular space. Asterisks mark unlabeled "vacuoles." The large arrow points at characteristic growth cone vesicles (most are <180 nm in diameter), which are not labeled. Magnification, 61,800x. Bar, 0.5 μm.

Table II. Morphometric Analysis of GCPs

| Condition | Vesicles (<180 nm) | Vacuoles (>180 nm) | GCPs |
|-----------|-------------------|-------------------|------|
|           | S (ves)           | V (gcp)           | S (vac) | V (gcp) | S (gcp pm) | V (gcp) |
| Control   | 0.087 ± 0.013     | 0.077 ± 0.014     | 0.24 ± 0.048 | 0.88 ± 0.12 | 1.20 |
|           | 6.4%              |                   | 20.1%    | 73.5%    | 100% |
| A23187    | 0.054 ± 0.015     | 0.048 ± 0.016     | 0.24 ± 0.033 | 0.89 ± 0.12 | 1.18 |
| % of S (total) | 4.1%              |                   | 20.4%    | 75.6%    | 100% |
| High K⁺   | 0.051 ± 0.012     | 0.040 ± 0.012     | 0.23 ± 0.031 | 0.84 ± 0.06 | 1.11 |
| % of S (total) | 3.6%              |                   | 20.7%    | 75.7%    | 100% |

GCPs were incubated at 37°C for 10 min with or without A23187 (10 μM) or for 5 min with high K⁺ (25 mM) before being fixed and processed for EM. 100 pictures were taken randomly at equivalent depths throughout each block and were analyzed blind with respect to condition (control, A23187 or high K⁺) at a final magnification of 51,000X. Volumes (V) and membrane areas (S) were estimated using standard stereological methods, i.e., point and intercept counting. Shown are the ratios of counts made for GCPs, their plasma membrane (pm) and the membrane of their vesicular structures (<180 nm vs those >180 nm in diameter). Other membrane compartments are so small that they do not contribute measurably to the total membrane pool, S (total), of GCPs. The S/V ratio for unidentified structures, such as lysed or tangentially sectioned elements, in the same samples are 1.46 ± 0.14, 1.49 ± 0.19, and 1.36 ± 0.15 for control, A23187 and high K⁺, respectively. The values indicate means ± SD of four determinations carried out on sections derived from two different blocks for each condition.

Isolated, sheared-off growth cones can be prepared sealed and kept viable for at least 30 min at 37°C as indicated by (a) the exclusion of inulin and ATP; (b) the absence of measurable LDH leakage in control and depolarizing conditions; (c) the lack of HRP influx in control and Ca²⁺-influx conditions; (d) continued ATP synthesis; (e) maintenance of ion gradients across the plasmalemma; and (f) Na⁺-dependent transmitter uptake into an osmotically sensitive compartment that is resistant to K⁺ depolarization and Ca²⁺ ionophore. Thus, intact GCPs offer an ideal, specialized system in which to investigate the phenomenon of plasmalemmal expansion separated from the synthetic functions of the cell.

Externalization of WGA Receptors and Identification of the Internal Membrane Pool

Our binding studies indicate the presence of a sizeable internal pool of WGA receptors (50% or greater of total sites) that...
of ~150 nm diameter, characteristic of growth cones (Pfenninger et al., 1983). Nevertheless, of the membrane compartments analyzed, only the <180-nm vesicles change significantly (reduction of ~40%) under conditions that externalize WGA sites. Therefore, lectin binding and morphometric data are consistent and demonstrate the externalization of an internal membrane compartment in GCPs. Furthermore, morphometry identifies the clear vesicles characteristic of growth cones as the plasmalemmal precursor (see Pfenninger and Bunge, 1974; Pfenninger and Maylie-Pfenninger, 1981; Cheng and Reese, 1987; Shea and Sapirstein, 1988).

Regulated Plasmalemmal Expansion Versus Transmitter Release

While the incorporation of new components into the plasmalemma is believed to be a constitutive process in the non-neuronal cells analyzed (for review see Burgess and Kelly, 1987), our results show that plasmalemmal expansion at the growth cone is regulated by a Ca2+-mediated mechanism. The presence in growth cones of aggregates of plasmalemmal precursor vesicles is consistent with this finding and reminiscent of vesicle clusters near sites of exocytosis in cells capable of regulated secretion (see Burgess and Kelly, 1987). The finding that fusion of precursor membrane with the plasmalemma is Ca2+-mediated is consistent with our current knowledge of mechanisms of exocytosis and complements studies implicating Ca2+ in the regulation of neurite outgrowth. Some results indicate that Ca2+ is a growth promoter (Reboulleau, 1986; Schubert et al., 1978; Gundersen and Barrett, 1980; Anglister et al., 1982; Suarez-Isla et al., 1984; Goldberg, 1988), while other reports suggest that it is a growth suppressor (Lettourneau and Wessells, 1974; Bixby and Spitzer, 1984; Kostenko et al., 1983; Cohan et al., 1987; Lankford and Letourneau, 1989). These apparently conflicting observations may be reconciled by recent studies on cul-

Table III. Uptake of 125I-HRP

| Incubation conditions | Incubation times | dpm* | dpm* |
|-----------------------|------------------|------|------|
|                       | 5 min            | 30 min | |
| Control SK, 37°C      | 2,408 ± 64       | 2,425 ± 25 |
| A23187 (10⁻⁶ M) in SK, 37°C | 2,279 ± 13       | 2,400 ± 13 |
| Control SK, 4°C       | 2,318 ± 16       | 2,400 ± 6  |

* dpm 125I-HRP taken up by GCPs (50 μg protein) in the conditions listed. The values are means ± SEM of two separate experiments performed in triplicate.

are indistinguishable by Scatchard analysis from those on the plasma membrane. Approximately 25% of these internal WGA sites are externalized upon treatment with agents known to cause Ca2+ influx, including Ca2+ ionophore A23187, high K+, and veratridine. Scatchard analysis shows that A23187-induced increase of external WGA binding is not caused by modification of the affinity of binding sites but by an increase in their number (Bronx). Endocytosis has been known to cause Ca2+ influx, including Ca2+ ionophore A23187-induced increase of external WGA binding. Therefore, fusion of new membrane with growth cone plasmalemma appears to be energy or ATP independent. This contrasts with the ATP dependence of membrane fusion reported for some other systems (Woodman and Edwardson, 1986; Holtz, 1988; Nube and Lindau, 1988; Churcher and Gomperts, 1990), and may suggest that plasmalemmal expansion in the nerve growth cone is regulated differently.

GCPs contain various membrane systems that are a potential source of components for insertion into the plasmalemma. These include sparse cisternae of putative smooth ER, a few synaptic vesicles, and other vesicles of various sizes, especially a large population of clustered clear vesicles of ~150 nm diameter, characteristic of growth cones (Pfenninger et al., 1983). In the present study, vesicular structures are divided somewhat arbitrarily on the basis of size (larger or smaller than 180 nm diameter) in an attempt to separate bona-fide "growth cone vesicles" (<180 nm) from most of the larger cross-sectioned plasmalemmal invaginations and vacuole-like structures. Such "vacuoles" are not normally seen in optimally preserved growth cones in vivo or in culture. While the <180-nm vesicle pool (64% of total membrane in control GCPs) excludes most of these structures, it clearly underestimates the compartment size of the typical "growth cone vesicles" (37% of total membrane in GCPs directly fixed after gradient separation; Pfenninger et al., 1983). Nevertheless, of the membrane compartments analyzed, only the <180-nm vesicles change significantly (reduction of ~40%) under conditions that externalize WGA sites. Therefore, lectin binding and morphometric data are consistent and demonstrate the externalization of an internal membrane compartment in GCPs. Furthermore, morphometry identifies the clear vesicles characteristic of growth cones as the plasmalemmal precursor (see Pfenninger and Bunge, 1974; Pfenninger and Maylie-Pfenninger, 1981; Cheng and Reese, 1987; Shea and Sapirstein, 1988).

Figure 9. [3H]-GABA and [3H]-5-HT uptake and release. (A) (left) GCPs were incubated for 30 min at 37°C with [3H]-GABA in SK buffer, in the presence or absence of DABA (200 μM), or without extracellular Na+ (Na+-free SK buffer), or at 4°C. (right) Synaptosomes were incubated for comparison for 30 min at 37°C with [3H]-GABA in SK buffer in the presence or absence of DABA (200 μM). Shown are the means ± SEMs of three experiments. (B) (left) GCPs were incubated for 10 min at 37°C with [3H]-5-HT in SK buffer, in the presence or absence of 1 μM imipramine (IMP), 1 μM benzotropine (BZT), or 10 nM desmethyl imipramine (DMI), or without extracellular Na+. (right) Synaptosomes were incubated for 10 min at 37°C with [3H]-5-HT in SK buffer in the presence or absence of 1 μM IMP. Shown are the means ± SEMs of three experiments. IMP blocks only ~55% of the uptake into GCPs. Approximately 20% of the IMP-insensitive [3H]-5-HT uptake may be accounted for by the dopamine transporter as suggested by the slight inhibition by 1 μM benzotropine, an effective blocker of dopamine uptake into synaptosomes (Hytell, 1982). DMI at 10 nM, a concentration blocking noradrenaline uptake into synaptosomes almost completely (Hytell, 1982), is without effect on [3H]-5-HT uptake into GCPs. (C-F) Release of [3H]-GABA and [3H]-5-HT from GCPs. Ordinates indicate the proportion (%) of released radioactivity relative to that remaining in the GCPs at the time of release. Arrows indicate the start and end of superfusion with the various agents listed below. (C) [3H]-GABA release in response to saponin in SK buffer or H2O, and to high-K+ SK buffer in the presence or absence of extracellular Ca++. Note logarithmic scale of ordinate. (D) [3H]-5-HT release in response to H2O and to high-K+ SK buffer in the presence or absence of extracellular Ca++. (E-F) [3H]-GABA (E) and [3H]-5HT (F) release in response to 10 μM A23187 in the presence or absence of extracellular Ca++. Control for changing medium, Ca2+-free SK buffer being replaced by SK buffer.
tured Helisoma neurons which provide evidence for a narrow concentration range of free cytosolic Ca\(^{2+}\) for optimal neurite elongation (Mattson and Kater, 1987). Tolkovsky et al. (1990) have reaffirmed the importance of Ca\(^{2+}\) in neurite outgrowth but have questioned the need for Ca\(^{2+}\) transients. In our GCPs the resting Ca\(^{2+}\) level may be below that required for plasmalemmal expansion but would reach the optimal growth range upon treatment with agents causing Ca\(^{2+}\) influx.

GCPs possess specific uptake mechanisms for GABA and 5-HT but do not exhibit Ca\(^{2+}\)-dependent release of these transmitters under the conditions that induce externalization of WGA sites or trigger transmitter exocytosis in synaptosomes (see Bowyer et al., 1987). This result, at least for GABA, is in agreement with data on developing brain slices (Balcar et al., 1983, 1986) and on growth cones isolated at different postnatal ages (Lockerbie et al., 1985; Taylor and Gordon-Weeks, 1989), which show that Ca\(^{2+}\)-dependent transmitter release appears in the rat only in the second postnatal week. Furthermore, Hume et al. (1983) have reported “weak coupling” between stimulation and secretion in growing cholinergic axons. Therefore, transmitter release is dissociated from plasmalemmal expansion during neuronal differentiation. This may be explained by differential behavior of synaptic vesicles versus plasmalemmal precursor vesicles in the growth cone and/or storage of most of the transmitter in the cytoplasm or another nonreleasable compartment.

Overall, these experiments show Ca\(^{2+}\)-regulated and ATP-independent plasmalemmal expansion at the growth cone, dissociated from transmitter release. The cell-free assay developed for these studies will prove to be very useful for experiments designed to investigate the biochemical mechanisms underlying Ca\(^{2+}\)-mediated membrane insertion.

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