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GAP-43 augments G protein-coupled receptor transduction in *Xenopus laevis* oocytes

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ABSTRACT **The neuronal protein GAP-43 is thought to play a role in determining growth-cone motility, perhaps as an intracellular regulator of signal transduction, but its molecular mechanism of action has remained unclear. We find that GAP-43, when microinjected into *Xenopus laevis* oocytes, increases the oocyte response to G protein-coupled receptor agonists by 10- to 100-fold. Higher levels of GAP-43 cause a transient current flow, even without receptor stimulation. The GAP-43-induced current, like receptor-stimulated currents, is mediated by a calcium-activated chloride channel and can be desensitized by injection of inositol 1,4,5-trisphosphate. This suggests that neuronal GAP-43 may serve as an intracellular signal to greatly enhance the sensitivity of G protein-coupled receptor transduction.**

The neuronal growth cone directs neurite outgrowth by selecting specific pathways and targets in response to stimulatory and inhibitory signals in the local environment (1, 2). A single filopodial contact can reorganize the entire growth cone, suggesting the presence of a powerful amplification system, which is poorly understood but may involve intracellular Ca^{2+} in some cases (2, 3). GAP-43 (a neural-specific protein also called neuromodulin, B50, F1, pp46, and p57) is concentrated in neuronal growth cones and is expressed at high levels during neuronal development and during periods of regeneration (4–6). It has been suggested that GAP-43 modulates neurite elongation (4–6). There is some direct evidence that GAP-43 can alter cell shape: transfection of the protein into nonneuronal cells increases the propensity to form filopodia (7); intracellular antibodies to GAP-43 decrease neuritogenesis by neuroblastoma cells (8); and overexpression of GAP-43 increases the sensitivity of PC12 rat pheochromocytoma cells to differentiation by nerve growth factor (9). GAP-43 is not essential for neurite extension, since PC12 cells with markedly decreased levels of the protein extend processes (10).

At a molecular level, GAP-43 may modulate signal transduction cascades in the growth cone and in mature synaptic terminals and thereby alter nerve terminal shape, growth, and/or secretion. *In vitro*, GAP-43 binds calmodulin (11, 12) and actin (13), serves as a substrate for protein kinase C (14), and inhibits phosphatidylinositol 4-phosphate kinase (15). Whether one or more of these activities occur and are of functional significance within nerve terminals has not been verified.

GAP-43 can also activate purified G_o, a heterotrimeric GTP-binding protein which is a major component of the neuronal growth-cone membrane (16, 17). GAP-43 would be an unusual G-protein regulator because GAP-43 is an intracellular protein (18), whereas G proteins generally are activated by transmembrane receptor–extracellular ligand complexes (19). GAP-43 increases guanine nucleotide exchange and steady-state GTP hydrolysis by purified G_o with a mechanism similar to that of receptor–ligand complexes (17). However, GAP-43 stimulation of G_o and G_i is distinguishable from receptor activation by $\beta\gamma$ subunit independence, pertussis toxin insensitivity, and phospholipid independence (17). It is not known whether GAP-43 can affect G proteins in the cellular milieu, nor how this might alter the coupling of receptors to G proteins. Since GAP-43 and G protein-coupled receptors have mechanistic and sequence similarities (16, 17), it is conceivable that they might act either competitively or synergistically to modulate G-protein activity.

To explore whether GAP-43 affects intracellular G proteins, and how this might modify receptor activation of G proteins, we developed two experimental models. In one assay, the three purified proteins were reconstituted into phospholipid vesicles, and in the other, GAP-43 was injected into *Xenopus laevis* oocytes, cells where the opening of Cl^- channels serves as a sensitive indicator for G-protein stimulation of phospholipase C (20). In lipid vesicles, GAP-43 activated G proteins and enhanced the G-protein response to receptor agonists, and in oocytes, its injection dramatically increased current flow in response to G-protein stimulation.

MATERIALS AND METHODS

GAP-43 Preparation. The purification of GAP-43 has been described (17). The concentration of GAP-43 was determined by a dye-binding method (Bio-Rad). For some experiments, GAP-43 (20 mg/ml) was preincubated with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (2 mg/ml) for 1 hr at 30°C in 100 mM KCl/5 mM sodium Hepes, pH 7.5/0.1 mM dithiothreitol. Trypsin was then inactivated by adding soybean trypsin inhibitor (4 mg/ml). As a control for the presence of trypsin, a second sample was prepared with trypsin inhibitor added prior to the 30°C incubation. Trypsin-treated GAP-43 (1 μM) did not significantly increase the rate of guanosine 5′-[γ-[^{35}S]thio]triphosphate (GTP[γ^{35}S]) binding to purified G_o [8 ± 17% increase (n = 3)], as measured in ref. 17. In contrast, the trypsin inhibitor/trypsin-treated GAP-43 preparation remained active, increasing GTP[γ^{35}S] binding to purified G_o by 78 ± 7% (n = 3).

GAP-43, Muscarinic M_2 Receptor, and G_o Reconstitution. Recombinant human muscarinic M_2 acetylcholine receptors were reconstituted with purified bovine brain G_o in liposomes (21). GTPase activity was measured with 0.16 nM receptor,

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Abbreviations: GTP[γ^{35}S], guanosine 5′-[γ-[^{35}S]thio]triphosphate; IP_3, inositol 1,4,5-trisphosphate.

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4 nM G_o, the indicated concentrations of GAP-43, 30 nM [α-^{32}P]GTP, 1.5 μM GDP, 2 mM $MgCl_2$, and 1 mM EDTA at 30°C with or without 100 μM carbachol (22). The effect of GAP-43 was most prominent at 30°C, in the presence of GDP. The GTPase activity of receptor–G_o vesicles was not significantly increased by GAP-43 at 20°C.

***X. laevis* Oocyte Preparation and Electrophysiology.** Stage V and VI oocytes were removed from anesthetized *X. laevis* (Xenopus I, Ann Arbor, MI) and defolliculated with collagenase (2 mg/ml; GIBCO). The cells were stored at 18°C in 96 mM NaCl/2 mM KCl/1 mM $MgCl_2$/5 mM sodium Hepes, pH 7.6 (ND-96 solution), with 1.8 mM $CaCl_2$, 2.5 mM pyruvate, 100 units of penicillin per ml, and 100 μg of streptomycin per ml. $5HT_{1c}$ serotonin receptor mRNA was synthesized with phage T7 RNA polymerase from plasmid pM1C2.3-p7 (plasmid generously provided by L. Yu, Indiana University), and 40 nl of a 50-ng/μl solution was injected into oocytes 2–4 days prior to further experiments. All experiments, except acetylcholine application, utilized $5HT_{1c}$ receptor RNA-injected oocytes. Oocyte current responses were monitored at a membrane potential of −60 mV in a two-electrode voltage-clamp apparatus (Dagan TEV-200) perfused with ND-96 containing 0.3 mM $CaCl_2$ and, for brief periods, acetylcholine (0.5 μM) or serotonin (1 μM).

Oocyte Microinjection and GAP-43 Immunoblots. GAP-43 was pressure-injected into oocytes through 3-μm pipettes, and the injection volume was calibrated by videomicroscopic observation. For the lower concentration of GAP-43, 10 nl of 20 μM GAP-43 in 100 mM KCl/5 mM sodium Hepes, pH 7.5/0.1 mM dithiothreitol was injected, resulting in a final estimated concentration of 0.2 μM from a 200-fmol injection into the 1000-nl oocyte. The higher concentration of GAP-43 was 200 μM in the pipette, yielding 2 μM in the cell from a 2-pmol injection. For control, we injected an equal volume of buffer that first had been dialyzed against the GAP-43 protein preparation for >24 hr. Some experiments involved injection of 4 pmol of inositol 1,4,5-trisphosphate (IP_3), yielding an estimated intracellular concentration of 4 μM.

GAP-43 immunoblots were prepared as described (17) by using total oocyte protein from cells injected 0–2 hr previously.

RESULTS

Purified GAP-43 and Receptor Stimulate G_o Synergistically. As a first step to assess the interaction of GAP-43 and receptor with G proteins, we incubated phospholipid vesicles containing purified muscarinic M_2 receptor and G_o with GAP-43 and the receptor agonist carbachol. The regulation of G_o was monitored by the level of GTPase activity (Fig. 1). Carbachol alone doubled the steady-state GTPase function, and saturating concentrations of GAP-43 caused a 45% increase. The combined presence of carbachol and GAP-43 resulted in higher levels of GTPase than either agent alone, and the effects were at least additive, with a total increase of 175% from basal values. Thus, although the effects are small after reconstitution, GAP-43 and receptor appear to act in concert. In contrast, G-protein stimulation by the wasp venom peptide mastoparan (22) is competitive with receptor action. Mastoparan inhibits GTPase activity slightly when assayed under maximal receptor stimulation (data not shown).

GAP-43 Augments Receptor-Induced Channel Opening. In *X. laevis* oocytes, binding of extracellular ligand to transmembrane receptor activates a heterotrimeric G protein, causing stimulation of phospholipase C and an increase in IP_3. IP_3 releases Ca^{2+} from intracellular stores and this directly opens plasma membrane Cl^- channels (20). Injection of activated bovine brain G_o stimulates this cascade (23).

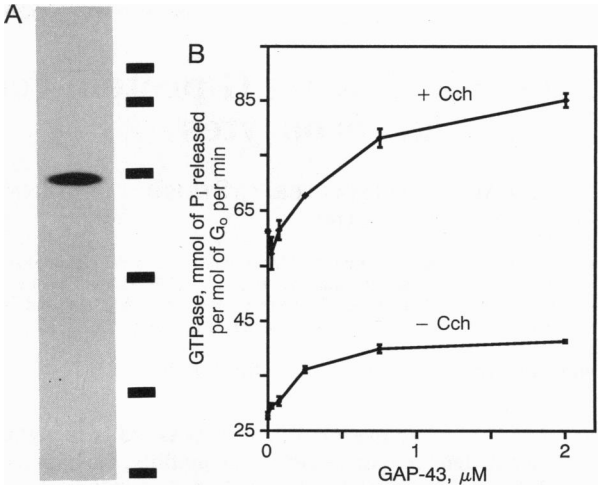

FIG. 1. GAP-43 and receptor stimulate G_o synergistically. (*A*) Purity of the rat brain GAP-43 preparation used in all experiments as shown by Coomassie blue staining of an SDS/polyacrylamide gel. Mobility of molecular size standards of 97, 66, 45, 31, 21.5, and 14 kDa is indicated at right. (*B*) GTPase activity of G_o/M_2 acetylcholine receptor vesicles as a function of GAP-43. GAP-43 increases GTPase to a greater extent in the presence (◆) than in the absence (■) of carbachol (CCh). The range of separate determinations for one preparation is illustrated. A second vesicle preparation yielded a 44% increase in GTPase with 4 μM GAP-43, a 290% increase with carbachol, and a 390% increase with GAP-43 plus carbachol.

Uninjected oocytes exhibited no detectable GAP-43 on immunoblots (Fig. 2). We microinjected oocytes with two different quantities of GAP-43. The lower intracellular GAP-43 concentration is about 1/12th that of whole neonatal rat brain (0.2 μM), and the greater concentration is equal to or slightly less than that in newborn rat brain (2 μM). An exact comparison of active GAP-43 concentrations is difficult because palmitoylation levels may vary between the oocyte and the brain, and this reversible posttranslational modification regulates GAP-43 stimulation of purified G_o (24).

The ability of receptor agonists to induce an inward current flow in voltage-clamped oocytes was compared before and after injection with buffer or GAP-43 (Fig. 3). Only one frog yielded oocytes with an endogenous acetylcholine response.

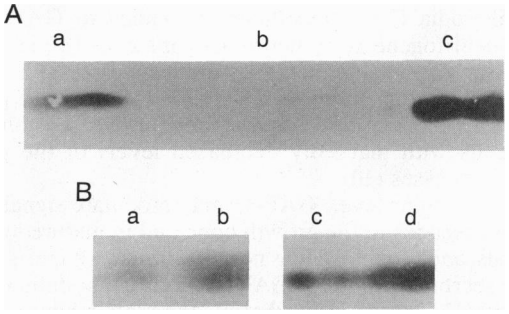

FIG. 2. GAP-43 injection into *X. laevis* oocytes. (*A*) GAP-43 immunoblot analysis demonstrates no GAP-43 in control oocytes (lane b, 240 μg of total oocyte protein), compared with strong staining of a neonatal rat brain sample loaded with much less total protein (lane c, 20 μg of total brain protein). After injection of oocytes with a low concentration of GAP-43 (lane a, 0.2 μM GAP-43, 240 μg of total oocyte protein), the level of GAP-43 was 1/12th or less than that of the brain sample. (*B*) Immunoblots for GAP-43 demonstrate that oocytes injected with a higher concentration of GAP-43 (2 μM GAP-43) contained approximately the same concentration of GAP-43 as neonatal rat brain by GAP-43 immunoblots. Lanes a and b, 10 μg and 20 μg of total oocyte protein; lanes c and d, 10 μg and 20 μg of brain protein.

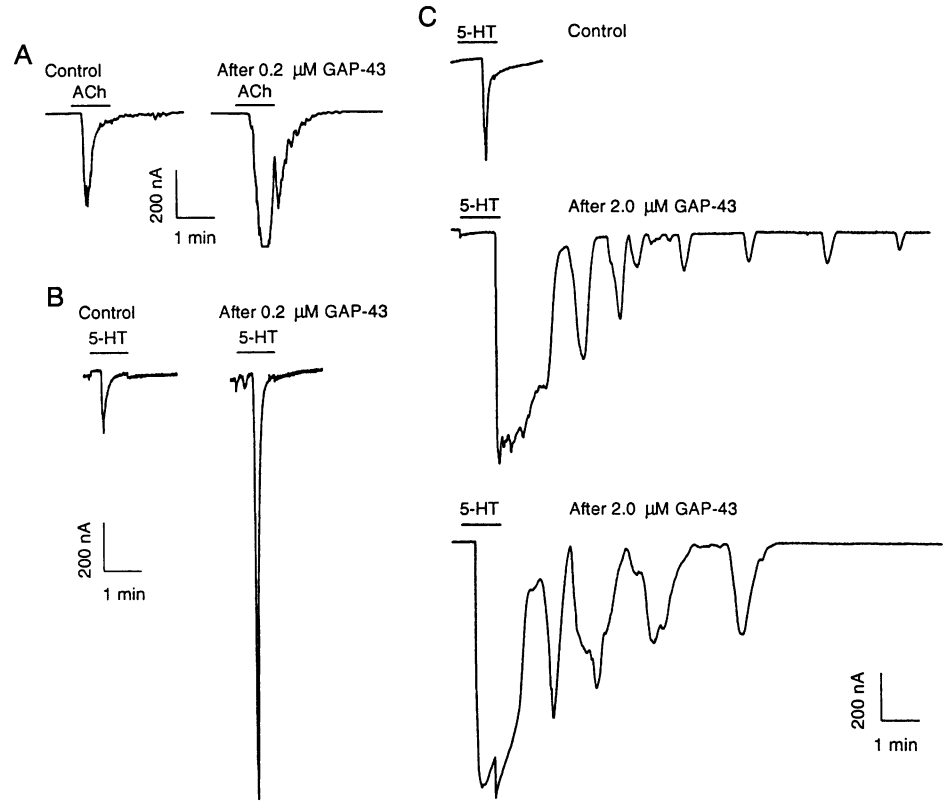

FIG. 3. GAP-43 augments G protein-coupled receptor action in *X. laevis* oocytes. (*A*) Current response of one oocyte to acetylcholine (Ach) before and after injection of the lower dose of GAP-43 (Fig. 2*A*). Note that the inward current response in this cell was enhanced after GAP-43 injection. Five of five cells exhibited a similar augmentation of acetylcholine responsiveness, with a 167 ± 36% increase (mean ± SEM, n = 5) in the total induced current flow after GAP-43 injection. In the right-hand trace, the peak current response saturated the voltage-clamp apparatus at 600 nA. (*B*) Serotonin (5-hydroxytryptamine, 5-HT) response of one oocyte before and after injection of GAP-43 as in Fig. 2*A*. After GAP-43 injection, the response was 2- to 5-fold greater. The response increased by >2-fold in 13 of 15 GAP-43-injected cells and in 1 of 18 buffer-injected control cells. (*C*) Response of three separate $5HT_{1c}$ receptor mRNA-injected oocytes from the same frog was recorded 20–100 min after injection with buffer (an average control response) or the concentration of GAP-43 in Fig. 2*B* (2 μM final GAP-43 concentration; two typical cells). The 5-HT response was markedly enhanced in all 11 GAP-43-injected cells examined in this paradigm.

GAP-43 injection (0.2 μM) more than doubled the total current flow induced by acetylcholine in these cells. No oocytes from 12 other frogs exhibited a response to acetylcholine, so we expressed an exogenous receptor, the rat $5HT_{1c}$ serotonin receptor, by injecting *in vitro* transcribed mRNA 3 days before analysis. The magnitude of the serotonin-induced current in these cells was not altered by buffer injection but was increased 2- to 5-fold by the lower GAP-43 concentration and >10-fold by the higher concentration of GAP-43 (Fig. 3).

GAP-43 Causes Opening of Ca^{2+}-Activated Cl^- Channels. In addition to facilitating receptor responses, GAP-43 by itself had a transient stimulatory effect on Ca^{2+}-mediated Cl^--channel opening (Fig. 4). The lower concentration of GAP-43 exhibited this effect only after prior stimulation with serotonin. When injected 4–10 min after a serotonin response, it caused an oscillating inward current of 10–250 nA which lasts for 3–10 min. This same GAP-43 concentration elicited no response from naive oocytes. The sensitization of the oocyte to GAP-43 by serotonin may be explained by previous observations that, during the period after receptor-induced whole-cell currents have ceased, waves of Ca^{2+} reverberate within the oocyte (25) and the response to IP_3 injection is exaggerated (26).

The higher GAP-43 concentrations produced a 5- to 30-min period of oscillating inward current, even without prior receptor stimulation (Fig. 4*B*). The peak amplitude of this response varied among different oocytes, from 30 to 2500 nA. A similarly prolonged response is known to occur after stimulation of certain G protein-coupled receptors in the oocyte (27). The ability of GAP-43 to induce this response was trypsin-sensitive, indicating that a protein component of the preparation was responsible. None of five cells exhibited an inward current response ≥ 20 nA after coinjection of 1 μM (final concentration) trypsin-treated GAP-43 and trypsin inhibitor; five of five oocytes responded to injection of 1 μM GAP-43 (final concentration) mixed with the trypsin inhibitor prior to trypsin exposure.

By several criteria, the GAP-43 response appeared to be mediated via the same G-protein cascade which is activated by receptors. The reversal potential for the GAP-43-induced current was −21 to −24 mV (data not shown), which is the reversal potential for Cl^- in the oocyte (28). The response to GAP-43 was abolished when the oocytes were coinjected with EGTA, as predicted for a Ca^{2+}-mediated event (Fig. 4). Injection of IP_3 caused a large inward current and was followed by refractoriness to application of IP_3, receptor agonist, or activated G protein for at least 1 hr (23, 29). IP_3 (4 pmol) also blocked the response to subsequent high concentrations of GAP-43 for up to 2 hr (four of four cells; data not shown). This desensitization was reversible, and the response to GAP-43 returned when examined 6 hr later. IP_3 is known to have no effect on the current response to injected Ca^{2+} (29). Thus, GAP-43 must act at a level of the cascade upstream of intracellular Ca^{2+} concentration. Although it is conceivable that the GAP-43 effect is due to prevention of a desensitization process induced by IP_3, this seems unlikely because desensitization to both IP_3 and receptor agonists occurred normally when GAP-43 was injected before IP_3 (Fig. 5).

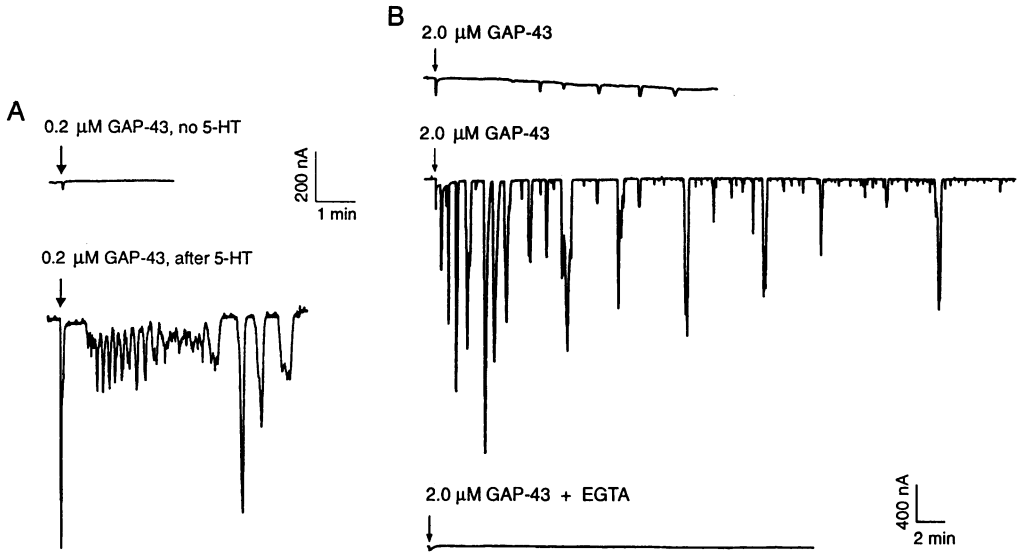

FIG. 4. GAP-43 stimulates Ca^{2+}-activated Cl^--channel opening. (*A*) Injection of the lower dose of GAP-43 (0.2 μM final concentration) produced no inward current in oocytes which had not been exposed to serotonin (5-HT) (upper trace; five of five cells). Injection of the same concentration of GAP-43, but 5–10 min after a 1-min exposure to 5-HT, resulted in current fluctuations (bottom trace; four of four cells). (*B*) Higher concentrations of GAP-43 (2 μM final concentration, as in Fig. 2*B*) resulted in an oscillating inward current response (top trace, the smallest positive response, and middle trace, a typical response from 23 positive cells of 25 injected cells). This type of spontaneous current was never seen in >12 hr of recording from more than 20 different buffer-injected and noninjected cells. Coinjection of EGTA with GAP-43 prevented the response (bottom trace, 0 of 12 cells with positive response). The concentration of EGTA was 50 mM in the pipette, resulting in an estimated 0.5 mM intracellular concentration after injection.

DISCUSSION

These data demonstrate that intracellular GAP-43 can both augment receptor activation of a G-protein transduction cascade and directly stimulate the same system. It is known that, as isolated proteins, GAP-43 stimulates the α subunit of G proteins (Fig. 1; refs. 16, 17, and 24). We assume that this is a site of interaction within the oocyte as well but cannot rigorously exclude other potential interactions, such as a GAP-43 action upon phospholipase C, G protein-coupled receptors or IP_3 receptors. There are, however, no biochemical data to suggest that GAP-43 can interact with any of these other proteins. GAP-43 does have other known biochemical effects, including interaction with cytoskeletal elements (13, 30) and neurotransmitter release (31), but these are unlikely to account for the effects noted here. GAP-43 binds calmodulin (11, 12), but this GAP-43 effect would be predicted to be downstream of Ca^{2+} release and so should not have been desensitized by prior IP_3 injection. GAP-43 can inhibit phosphatidylinositol 4-phosphate kinase (15), but this interaction would be predicted to inhibit rather than stimulate the oocyte IP_3-dependent cascade. Thus, these experiments show that GAP-43 can affect the G-protein cascade in the cellular milieu and suggest that it does so at the level of the G protein or receptor–G protein coupling.

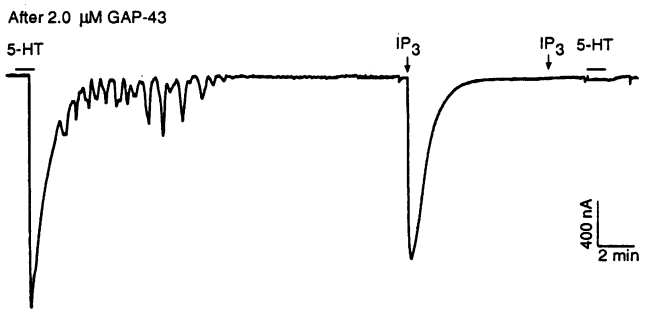

FIG. 5. GAP-43 does not block IP_3-induced desensitization. A continuous current trace from one oocyte 30 min after injection with the high concentration of GAP-43 (2 μM) is shown. Serotonin (5-HT) produced a large response which returned to baseline after 9 min; this was followed by a current response to the intracellular injection of 4 pmol of IP_3. Eight nanoliters of 0.5 mM IP_3 (Sigma; estimated intracellular concentration, 4 μM) in water was injected by the same method as for GAP-43. Subsequent bath application of 5-HT or intracellular injection of IP_3 in this GAP-43-injected oocyte produced no change in current. Similar results were observed in three of three cells examined in this protocol.

GAP-43 facilitation of receptor agonist action occurs at low GAP-43 concentrations, so this may be the more prominent effect *in vivo*, as opposed to direct stimulation of G proteins. GAP-43 could therefore be considered an intracellular modulator of the sensitivity, or gain, of G protein-coupled receptor transduction. In general, regulation of G-protein cascades occurs through transmembrane receptors for extracellular ligands (19), and so GAP-43 control of G protein function could provide another facet to this system. The recent description of phosducin as a regulator of heterotrimeric G proteins is another example of intracellular modulation of G protein-activity (32). The more-than-additive effect of GAP-43 and receptor stimulation of G protein argues that the two proteins associate with different sites of the G protein. These two G-protein modifiers do not appear to compete for the same site.

GAP-43 and G_o are both highly enriched in neuronal growth cones (16, 33, 34). If an interaction similar to that in oocytes were to occur in the growth cone, it could alter growth-cone motility, since G-protein activation appears to be an important regulation of that structure. Stimulation or inhibition of G proteins with nonhydrolyzable guanine nucleotides alters neurite outgrowth from embryonic sympathetic neurons (6). Mastoparan, which activates heterotrimeric G proteins of the G_o and G_i subtypes, induces growth-cone collapse in embryonic dorsal root ganglion cells (35). G protein-coupled receptors for the neurotransmitters serotonin (36), dopamine (37, 38), and glutamate (39) can cause growth-cone collapse, and the G protein-coupled receptor for the protease thrombin induces neurite retraction (40). Cell adhesion molecules may also alter growth cone motility by a G protein-dependent mechanism. Antibodies to L1 and

NCAM cell adhesion molecules alter PC12 cell inositolphospholipid and Ca^{2+} levels in a pertussis toxin-sensitive fashion (41). The morphoregulatory effects of NCAM and N-cadherin are blocked by pertussis toxin treatment (42). A group of membrane-bound target-derived factors have been described and are thought to collapse growth cones at appropriate synaptic targets (43–46). Growth-cone collapse induced by these factors is blocked by pertussis toxin, implicating a G-protein signaling pathway (35). The expression of GAP-43 in the growth cone might serve to enhance the response to these G protein-dependent extracellular signals. The cellular effect of GAP-43 action would depend upon the context of the specific neuron and its environment, so that a GAP-43-enhanced signal might increase the forward motion of the growth cone in some cases and decrease it in others.

The present study supports the hypothesis that GAP-43 alters growth-cone motility by regulating G-protein activation (6). The localization of GAP-43 to the neuronal growth cone may reflect a requirement for high-level amplification of G protein-mediated signals derived from a single filopodial contact. G-protein regulation might also be a unifying principle for other proposed cell biologic roles of GAP-43, such as the control of neurotransmitter release (31) and synaptic plasticity (4, 47, 48) of mature neurons. The activity of heterotrimeric G proteins is known to contribute to the regulation of neurotransmitter release (49), and pertussis toxin can block the generation of long-term potentiation in hippocampal slices (50).

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