

Rab effector domain peptides stimulate the release of neurotransmitter from cell cultured synapses

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Received 29 April 1993

The involvement of the small GTP-binding protein rab3A in synaptic transmission was tested by presynaptic microinjection of guanine nucleotides and peptides corresponding to the effector domain of rab3A. When GTP γ S injection was paired with presynaptic action potentials, the frequency of MIPSCs was increased and the evoked synaptic current was reduced in magnitude. To more specifically manipulate rab proteins, peptides were microinjected. Injection of the peptide rab3AL(33–48) or rab3(29–48) stimulated an increase in the frequency of MIPSC with little effect on action potential evoked synaptic transmission supporting a role for rab proteins in regulating synaptic transmission.

Rab3A; Rab peptide; Exocytosis; Calcium; Synapse; Small GTP-binding protein

1. INTRODUCTION

Small GTP-binding proteins play critical roles in vectorial membrane transport between cellular compartments [1,2]. The small GTP-binding protein rab3A has emerged as a candidate in the regulation of vectorial transport of synaptic vesicles from a vesicle storage pool to docking sites at the plasma membrane in the nerve terminal [1–4]. Rab3A, which is found exclusively in secretory cells, is associated with synaptic vesicles [5,6]. In addition to its critical localization in the synapse, rab3A dissociates from the vesicular membrane following potassium-induced depolarization of synaptosomes [5]. Rab3A-GDP binds to a GDP dissociation inhibitor (GDI) which promotes the separation of rab3A from the vesicular membrane [7]. Thus, rab3A associates and dissociates from synaptic vesicles during synaptic transmission indicating that it may play a regulatory role in this process.

Previous studies have probed the role of GTP-binding proteins in synaptic transmission through the microinjection of guanine nucleotide analogs [8–10]. Presynaptic microinjection of non-selective G protein activators, such as the non-hydrolyzable analog of GTP, GTP γ S, resulted in reduced action potential-evoked synaptic transmission. However, since GTP γ S is likely to activate many GTP-binding proteins these data have to be interpreted with caution. Using giant synapses which form between contacting somata of cell-cultured

neurons we have compared the effects of GTP γ S with more selective probes for rab protein involvement, specifically, peptides corresponding to the putative effector domain of rab3A [11]. This latter approach has been used successfully to demonstrate that synthetic peptides of the rab3A effector domain inhibit ER to Golgi and intra-Golgi transport [11]. Rab3A peptides have also been shown to stimulate secretion in mast cells [12], pancreatic acini [13], and chromaffin cells [14]. In this study we demonstrate that microinjection of both GTP γ S and rab peptides into neurons increase the spontaneous frequency of miniature synaptic currents. In contrast, rab peptides have no significant effects on action potential-evoked release of neurotransmitter in the same synapses. These data provide experimental support for the hypothesis that rab GTP-binding proteins play regulatory roles in the synapse. Some of this work has appeared in preliminary form [15].

2. MATERIALS AND METHODS

Cultured neurons were obtained from adult specimens of the albino snail *Helisoma trivolvis*. Identified neurons B5 and VD4 were isolated from the buccal and visceral ganglia, respectively, according to previously published techniques [8,16,17]. In brief, the central ganglia were removed from the animal, incubated for 15 min at room temperature in 0.2% trypsin (Sigma type III) in defined medium (DM; 50% Liebowitz-15 (L-15-Gibco) with additional *Helisoma* salts; [16]). Neurons were removed from the ganglia by applying suction to a heat-polished macropipette. Pairs of neurons B5 and VD4 were plated in culture and maneuvered together to allow adhesion of cell bodies using positive pressure from the transfer macropipette. Typically four neuron pairs were plated in each Falcon # 1008 plastic Petri dish within a 75 ml drop of conditioned medium (CM; [16]) to which 5 μ l of *Helisoma* blood extract was added. Blood collected from donor snails was centrifuged at 45,000 rpm for 90 min. Following centrifugation the upper

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clear layer of the blood sample was decanted leaving a viscous red blood extract which was stored at -70°C prior to use. Synaptic pairs were left in these culture conditions for two days without exchanging medium. After two days cell pairs were transferred to a recording chamber consisting of a #1008 Petri dish in which a poly-L-lysine treated coverslip had been attached to the hollowed out base. Under these conditions the neuronal somata adhere to the glass coverslip and become immobilized which is necessary for electrophysiological and fluorescent examination.

2.1. Presynaptic intracellular recordings

Presynaptic neuron B5 was penetrated with an intracellular glass fiber-filled microelectrode which served to monitor and control the neuronal activity of B5 and to microinject test solutions. The electrophysiology of B5 was monitored using a Dagan 8800 amplifier and stored on VHS tape using a Vetter PCM video recorder. When necessary a hyperpolarizing current bias was applied to B5 to prevent spontaneous firing. In experiments where action potentials were required, neuron B5 was stimulated to produce one action potential every 30 s using either UMANS software (Chester M. Regan, Urbana, IL) when simultaneous fluorescence measurements were taken, or through pClamp software (Axon Instruments, CA).

2.2. Postsynaptic voltage clamp

Postsynaptic neuron VD4 was whole-cell voltage clamped using a patch pipette with a DC resistance of 1–2 M Ω via an Axopatch-1C (Axon Instruments, CA). The postsynaptic pipette solution consisted of (in mM): 50 KCl, 5 MgCl₂, 5 EGTA and 5 K⁺-HEPES at pH 7.3. Neuron VD4 was voltage clamped at a holding potential of -80 mV. At this potential and with the inclusion of 50 mM KCl, postsynaptic events in neuron VD4 are seen as inward currents. Synaptic currents were monitored in neuron VD4 and stored on VHS tape using the Vetter PCM video recorder. MIPSCs were counted in 30 s time bins to obtain measures of MIPSC frequency. In all experiments a 3 min recording period was monitored prior to microinjection. Cell pairs exhibiting an average MIPSC frequency of $< 2 \text{ min}^{-1}$ during the pretest period were discounted. If the MIPSC frequency in the first 30 s bin following microinjection was 2 times or greater the pretest average MIPSC frequency, these cell pairs were rejected since this reflected cell damage due to injection.

2.3. Presynaptic injections

Microinjections of B5 were performed using a Narishige IM-200 (Narishige USA, Inc.). The presynaptic microelectrode was filled with the test solution (tip diameter, 2 μm). GTP and GTP γ S were microinjected into neuron B5 at a concentration of 2 mM in a solution of (in mM): 60 KCl, 0.2 EGTA and 10 HEPES, pH 7.3. Typically microinjection of guanine nucleotides required one injection pulse (30–50 ms) at 15 PSI.

Peptide stock solutions were prepared at 10 mg/ml in a solution of (in mM): 50 KCl, 5 EGTA and 10 HEPES. To facilitate microinjection the stock solutions of peptides were diluted 3.3 mg/ml with a solution of (in mM): 50 KCl and 10 HEPES. Fluorescein was added to the injection solution to permit calibration of the volume of the electrode solution injected and thus the concentration of peptide in the presynaptic cell. The single letter amino acid code of peptides injected are: rab3AL(33–48) VSALGIDFKVKTIYRN; rab3(29–48) TPAFVSTV-GIDFKVKTIYRN, and ral EPTKADSYRKKVVLVD.

2.4. Chemicals

GTP and GTP γ S lithium salts were obtained from Boehringer Mannheim Biochemicals. Fura-2 was obtained from molecular probes and synthetic peptides were provided by W. Balch.

All data are presented as mean \pm S.E.M. Student's *t*-test was used to assess statistical significance.

3. RESULTS

To experimentally perturb synaptic proteins it is nec-

essary to have local access to the presynaptic terminal. To achieve this end we constructed a chemical synapse between pairs of spherical neurons B5 and VD4 as previously described for other *Helisoma* synapses [18,19]. Neurons were plated together in a non-adhesive culture environment where soma–soma adhesion was maintained in the absence of neurite extension or adhesion to the culture. In this configuration somata formed chemical synapses with similar properties to the synapse which forms between neurites of the same cell types [20]. Action potentials in B5 evoked 1 for 1 inhibitory postsynaptic currents (IPSCs) in neuron VD4 which were blocked by removing external calcium from the bathing medium and were reduced in amplitude by addition of the cholinergic antagonist, tubocurarine chloride (10 mM; not shown). At a postsynaptic holding potential of -80 mV and with elevated Cl[−] concentration (50 mM) of the postsynaptic recording solution, IPSCs are detected as inward currents in neuron VD4.

3.1. GTP γ S augments miniature frequency but depresses evoked release of transmitter

GTP γ S, the non-hydrolyzable analog of GTP, was microinjected into the presynaptic cell to irreversibly activate the cell's G proteins which include heterotrimeric G proteins and small GTP-binding proteins. The effects of GTP γ S on synaptic transmission were complex and dependent on synaptic activation (Fig. 1). Initially, the action of GTP γ S was monitored by measuring the frequency of spontaneous miniature inhibitory synaptic currents (MIPSCs) in the absence of presynaptic action potentials. MIPSC frequency was recorded for a control period of 3 min and all values were normalized to the mean MIPSC frequency during the control period. Microinjection of GTP γ S in these experiments caused a small reduction in the frequency of spontaneous MIPSCs, as previously reported [8] (Fig. 1A) from a control value of 1.0 to 0.69 ± 0.23 ($n = 9$) within 6 min after injection. Previous studies indicate that this action is likely to involve the activation of heterotrimeric, pertussis toxin-sensitive (PTX) G proteins which have been demonstrated to reduce the secretory response to internal calcium [8].

When GTP γ S was microinjected into synapses while evoking presynaptic action potentials at intervals of 30 s to 1 min the frequency of MIPSCs increased. Three minutes following GTP γ S injection MIPSC frequency increased greater than two-fold (Fig. 1A; $n = 6$). This elevated MIPSC frequency is significantly greater than the MIPSC frequency detected when GTP ($P < 0.01$; $n = 6$) was injected into synapses or when GTP γ S was injected into unstimulated synapses (Fig. 1A; $P < 0.01$; $n = 9$).

While GTP γ S enhanced MIPSC frequency, the magnitude of action potential evoked IPSCs in the same synapses was decreased (Fig. 1B). GTP-injection did not significantly modify the amplitude of evoked IPSCs

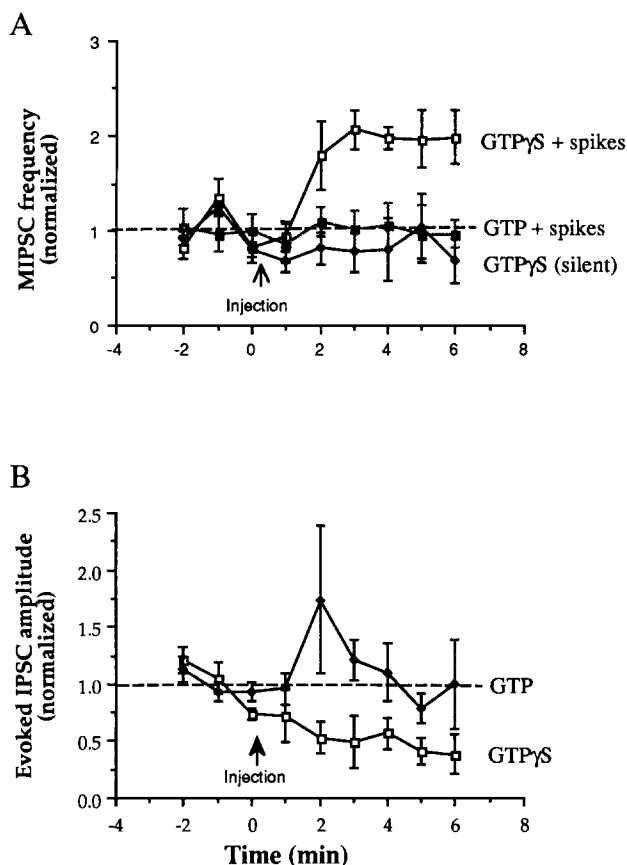


Fig. 1. GTP γ S has multiple actions on synaptic transmission. Synapses were microinjected with either GTP γ S or GTP and the effects on MIPSC frequency (A) or action potential evoked synaptic transmission (B) were monitored. (A) GTP γ S causes a small reduction in MIPSC frequency when injected into resting synapses. However, when injected into active synapses, GTP γ S causes an acceleration of MIPSC frequency. GTP injection in active synapses does not affect MIPSC frequency. (B) GTP γ S injection reduces the magnitude of the action potential evoked synaptic current while GTP has no significant effect.

(Fig. 1B). While at apparent odds with the action of GTP γ S on MIPSC frequency, this result is expected since PTX-sensitive G proteins are known to reduce calcium influx in synapses formed by neuron B5 [8]. These data reveal a conditional action of GTP γ S on MIPSC frequency which requires simultaneous presynaptic action potentials and the presentation of GTP γ S.

3.2. Rab3 peptides selectively increase the frequency of MIPSCs without affecting action potential evoked release of transmitter

To test whether the GTP γ S-induced increase in MIPSC frequency might be mediated through rab-like GTP-binding proteins we determined the effects of synthetic peptides on synaptic transmission. Peptides with sequences spanning the putative effector domain of rab3A were microinjected into the presynaptic neuron B5 while MIPSC frequency and action potential-evoked synaptic transmission were monitored. Initially a 16

amino acid peptide rab3AL(33–48) was microinjected. This peptide corresponded to the amino acid residues 33–48 of the putative effector domain but contained two amino acid substitutions, A and L, at positions 35 and 36. Previous studies have demonstrated that this is a potent peptide for perturbing rab function [11]. Fig. 2 shows the effect of rab3AL(33–48) on MIPSC frequency. Within minutes after microinjection of rab3AL(33–48) MIPSC frequency increased greater than two-fold. This increased MIPSC frequency was not accompanied by a significant change in unitary amplitude of the MIPSCs. Fig. 2C shows amplitude distributions of MIPSCs shown in Fig. 2B. Prior to peptide injection, the MIPSCs have a mean amplitude of 15 pA. Following rab3AL(33–48) injection, the number of MIPSCs detected at this amplitude increased, and events twice the unitary amplitude were recorded, presumably because of the synchronous release of two quanta. Pharmacologically, MIPSCs were similar before and after peptide injection since the cholinergic antagonist tubocurarine was demonstrated to reduce the magnitude of MIPSCs in uninjected control preparations as well as following injection of the peptide rab3AL(33–48).

The augmentation of MIPSC frequency is dose-dependent (Fig. 3A). Over the range of rab3AL(33–48) concentrations tested (0–60 mM), the maximal MIPSC frequency increase was three-fold the pre-injection level. In contrast to the stimulatory action on spontaneous MIPSC frequency, rab3AL(33–48) did not significantly change the magnitude of action potential evoked IPSCs (Fig. 3B). Thus, the peptide rab3AL(33–48) selectively augments MIPSC frequency without affecting the amplitude of evoked IPSCs.

Rab3AL(33–48) might increase MIPSC frequency through actions on internal calcium or on the secretory machinery. To determine whether peptide injection caused a detectable increase in presynaptic resting calcium, B5 somata were injected with Fura-2 and the subsequent actions of rab3AL(33–48) were determined. Fura-2 was pressure injected into B5 somata to yield a final concentration of about 100 μ M. Neurons were then re-impaled with a second electrode which contained rab3AL(33–48). Rab3AL(33–48) injection caused a small insignificant reduction in resting calcium levels of -4 ± 8 nM ($n = 4$). While significant elevations of internal calcium were not detected using Fura-2 it is possible that rab3AL(33–48) caused a tonic calcium influx that was restricted to a sub-plasma membrane domain and was undetected by Fura-2. To test for this possibility, synapses were recorded from, peptide was injected to augment MIPSC frequency and then external saline was changed to a high Mg^{2+} /zero Ca^{2+} saline. When external calcium was removed from the bathing medium, action potential evoked synaptic transmission was immediately blocked without a simultaneous reduction in the enhanced MIPSC frequency ($n = 2$). Thus,

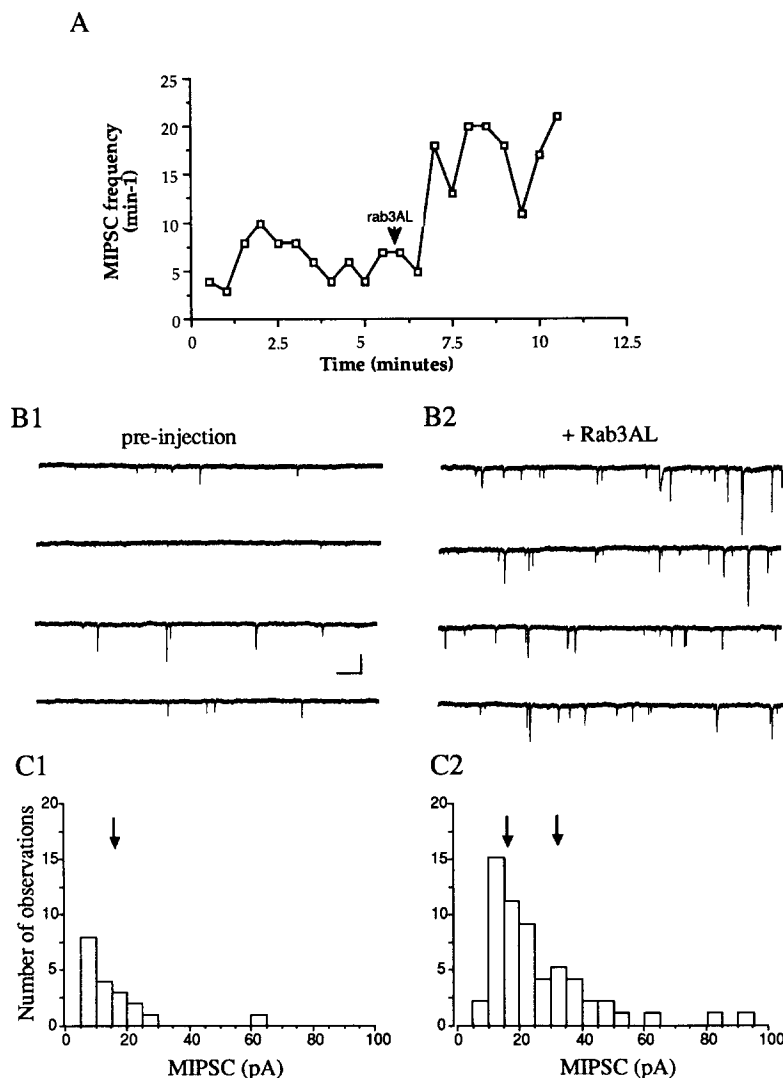


Fig. 2. Microinjection of rab3AL(33–48) accelerates the frequency of MIPSCs. (A) shows the frequency of MIPSC in a single preparation. At the arrow, rab3AL(33–48) injection caused an rapid increase in MIPSC frequency which was sustained for the period of recording. (B) shows examples of the MIPSCs recorded before (B₁) and after rab3AL(33–48) injection (B₂). (C) Shows amplitude distribution histograms for the MIPSCs before (C₁) and following (C₂) rab3AL(33–48) injection. Arrow (C₁) denotes the mean amplitude of MIPSCs during the recording period, and in C₂ the two arrows mark the amplitudes of 1 and 2 quanta derived from the data prior to peptide injection. Recordings were made from postsynaptic neuron VD4 in the whole cell configuration (V_{hold} -80 mV; see section 2). Calibration in B: 20 pA and 1 s.

these data support the notion that rab3AL(33–48) directly regulates the secretory process to increase MIPSC frequency.

To test the specificity of rab3AL(33–48), other synthetic peptides were microinjected into the presynaptic neuron. The low solubility of many of the rab peptides prevented us from testing a variety of different peptide sequences. However, two additional peptides were successfully used. The first of these peptides, ral, is a member of the ras superfamily of small G proteins and has little homology with the putative effector domain of rab3A. Microinjection of ral did not significantly affect MIPSC frequency or the magnitude of action potential evoked IPSCs (Fig. 4A). A second peptide rab3(29–48) which spans the putative effector domain of rab3A and

contains no amino acid substitutions caused an increase in MIPSC frequency similar to that seen with rab3AL(33–48). Over the concentration range 20–40 mM the increase in MIPSC frequency in response to rab3(29–48) was 2.6 ± 0.62 (mean \pm S.E.M; $n = 8$) compared to 2.6 ± 0.61 ($n = 7$) for rab3AL(33–48) and 0.82 ± 0.18 ($n = 7$) for ral (Fig. 4A). MIPSC frequencies were significantly elevated following rab3AL(33–48) ($P < 0.02$) and rab3(29–38) ($P < 0.02$) injection as compared to ral injection. Over the same concentration range there were minor effects of peptides on evoked IPSC amplitude. IPSC was moderately increased to 1.15 ± 0.29 ($n = 5$) following rab3AL(33–48) injection, to 1.6 ± 0.29 ($n = 7$) when rab3(29–48) was injected, and to 1.15 ± 0.2 ($n = 4$) following injection of ral (Fig.

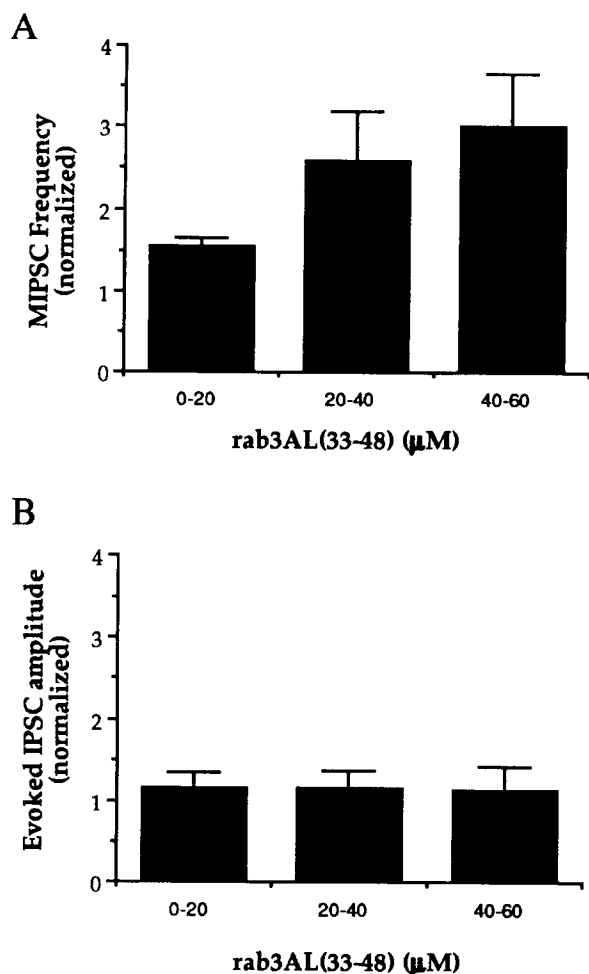


Fig. 3. Rab3AL(33-48) causes a dose-dependent increase in MIPSC frequency (A) while having no significant effect on the magnitude of evoked IPSCs in the same preparations (B).

4B). These data demonstrate that rab peptides specifically increase the basal frequency of exocytosis and have little effect on action potential evoked transmission.

We demonstrated that the stimulatory action of GTP γ S on MIPSC frequency required activity in the presynaptic neuron (Fig. 1). We therefore asked whether the stimulatory effect of rab3AL(33-48) on MIPSCs similarly required synaptic activity. The spontaneous frequency of MIPSCs was monitored while hyperpolarizing current was injected presynaptically to prevent the discharge of action potentials. Microinjection of rab3AL(33-48) reliably caused an increase in frequency of MIPSC even in the absence of presynaptic action potentials. Rab3AL(33-48) (20-40 μ M) increased MIPSC frequency from a normalized control frequency of $1.0-4.04 \pm 2.67$ within minutes of microinjection. This increase in MIPSCs was indistinguishable from the action of this peptide on MIPSCs when presynaptic activity was present. Thus, in contrast to our observations with GTP γ S, rab3AL(33-48) is able to

increase MIPSC frequency independent of presynaptic action potentials.

4. DISCUSSION

By microinjecting two classes of molecules into the presynaptic terminal this study has demonstrated roles for G proteins in regulating synaptic transmission. Using the non-selective G protein activator, GTP γ S, we have demonstrated conditional roles for G proteins in the synapse. In the absence of presynaptic action potentials, GTP γ S, reduces MIPSC frequency, an action that is mediated by heterotrimeric G proteins and underlies a FMRFamide dependent presynaptic inhibition of synaptic transmission [8]. However, when the synapse is in use, due to presynaptic action potentials, GTP γ S has an opposing effect and increases MIPSC frequency. This conditional effect may be due to calcium entry during the action potential, or because of a G protein dependent effect on vesicle mobilization and/or vesicle docking.

Other experimental perturbations of synapses with guanine nucleotides have produced complementary data to those described in this study. Hess et al. [10] have shown in the squid giant synapse and Baux et al. [9] have shown in *Aplysia* that GTP γ S injection reduces action potential-evoked synaptic transmission. Similar actions are observed in *Helisoma* synapses. In *Helisoma* and *Aplysia* this effect can be accounted for by GTP γ S activating heterotrimeric G proteins which regulate calcium influx and the secretory response to internal calcium [8]. However, in squid, GDP β S also reduces synaptic transmission suggesting the involvement of small G proteins in this effect [10]. Since miniature synaptic potentials were not monitored in the squid it is unclear whether GTP γ S was able to have a similar stimulatory effect on basal release of neurotransmitter.

Since GTP γ S generally activates GTP-binding proteins and causes multiple effects in the synapse we used peptides which perturb rab GTP-binding pathways to examine their role in the synapse rather than pursuing the further use of other guanine nucleotide analogs. Microinjection of rab peptides selectively increased MIPSC frequency without changing action potential-evoked IPSC magnitude. The action of peptides was sequence specific. Injection of the putative effector domain of ral did not augment MIPSC frequency. Since the rab3 effector domain is similar to the effector domains of other rab proteins our data do not discriminate between potential perturbations to other rab systems. Taken together with the extensive data demonstrating the presence and regulation of rab3A in synapses, our microinjection perturbation experiments provide experimental support for the hypothesis that rab proteins play regulatory roles in the synapse. However, further experiments are required to determine the precise role of these proteins.

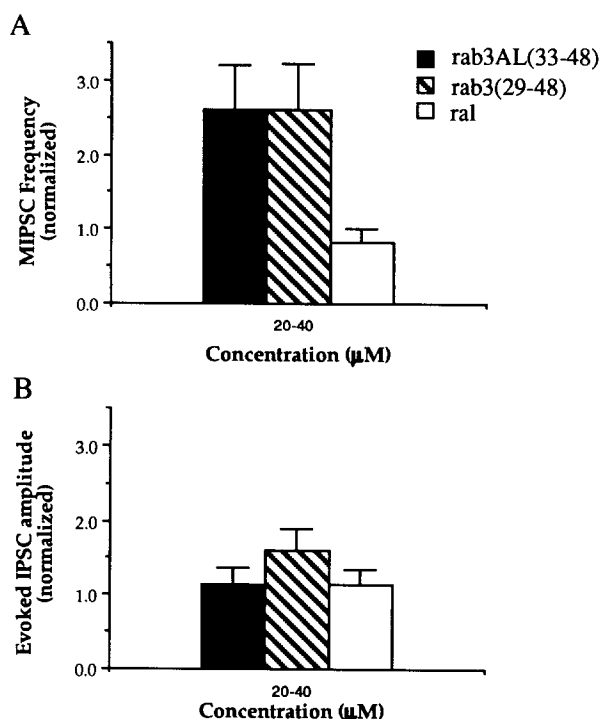


Fig. 4. Rab peptides selectively increase MIPSC frequency. Three different peptides were injected presynaptically into synapses. Rab3AL(33-48) and rab3(29-48) stimulate an increased frequency of MIPSCs while a ral peptide, with little sequence homology with the rab effector domain, does not significantly affect MIPSC frequency (A). In contrast to the stimulatory actions of rab peptides on MIPSC frequency, rab3AL(33-48) and rab3(29-38) have modest actions on the magnitude of the action potential evoked IPSC.

The stimulatory mechanism of rab peptide action in secretion [12-14] is not defined. By analogy with ras-GAP one explanation is that the peptides prevent rab-GAP interaction and thus reduce rab hydrolysis of GTP. Alternatively, the rab peptides may directly interact with a rab-effector protein (REP) to promote a stimulatory action. Studies by Oberhauser et al. [12] support the possibility that peptides stimulate a REP since the action of rab peptides is enhanced in mast cells when GTP is dialyzed out of the cell, or when GDP β S is added to the cell.

There are many explanations for the differential effects of rab peptides on MIPSC frequency and action potential evoked transmitter release. (1) Rab peptides might stimulate a calcium-independent pathway of quantal neurotransmitter release that is parallel to calcium regulated release. (2) Perhaps the exocytosis of vesicles that are distant from calcium channels is being stimulated by rab peptides. (3) Alternatively the peptide might act on an effector protein which responds to multiple stimuli including calcium and the rab effector domain. In this scenario, a differential action on MIPSC frequency and evoked transmission would require that rab peptide and calcium signals for exocytosis were inte-

grated in an additive manner by a common macromolecular complex. Under conditions of low internal calcium, in resting synapses, rab peptides could significantly increase the frequency of basal exocytosis. However, during a large calcium stimulus such as supplied by the action potential, the action of rab peptide would be small, and undetectable by comparison to the calcium-stimulus.

A comparison of the effects of GTP γ S and rab peptides on transmitter release is difficult because GTP γ S activates many GTP-binding proteins simultaneously. However, an important observation with the GTP γ S injection experiments is that both GTP γ S and presynaptic action potentials are required to promote the increased frequency of MIPSCs. While this may be due to an interaction between calcium entry during action potentials and GTP γ S, it is also possible that it is related to the recruitment of a vesicle associated GTP-binding protein, such as rab3A, during vesicle cycling. A difference between the GTP γ S and rab peptide data is that the peptides are able to increase MIPSC frequency in the absence of presynaptic action potentials while GTP γ S is not. This might be expected since a peptide may have ready access to the REP while GTP γ S might not since GTP γ S would only act at this site when associated with the rab3 protein and associated vesicle.

In conclusion, our data demonstrate that rab3A peptides with sequences spanning the putative effector domain of this protein cause a significant stimulation of basal release of neurotransmitter. However, action potential evoked release of neurotransmitter is unaffected by the same peptides. Similar differential effects on MIPSC frequency and action potential evoked synaptic transmission were also detected following microinjection of the non-hydrolyzable analog of GTP, GTP γ S. These data provide experimental data in support of a role for the small GTP-binding protein rab3A in the regulation of the synapse.

Acknowledgements: The authors would like to thank Dr. William Balch for his generous gifts of peptides used for this study and for his discussions during the course of this work. We are indebted to Dr. Julio Fernandez for his insight and help during this work. This work was supported by NIH Grants NS24233 and NS26650 and by the Alfred P. Sloan Foundation.

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