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Regulation of the Ca^{2+} Sensitivity of Exocytosis by Rab3a

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Abstract: Ca^{2+} ions trigger the release of hormones and neurotransmitters and contribute to making the secretory vesicles competent for fusion. Here, we present evidence for the involvement of the GTP-binding protein Rab3a in the sensitivity of the exocytotic process to internal [Ca^{2+}]. The secretory activity of bovine adrenal chromaffin cells was elicited by Ca^{2+} dialysis through a patch-clamp pipette and assayed by monitoring changes in cell membrane capacitance. Microinjection of antisense oligonucleotides directed to rab3a mRNA increased the secretory activity observed at low (0.2–4 μM) [Ca^{2+}], but did not change the maximal activity observed at 10 μM free [Ca^{2+}]. Moreover, after a train of depolarizing stimuli, the secretory activity of antisense-injected cells dialyzed with 10 μM [Ca^{2+}] was increased significantly compared with that of control cells. This result suggests that the activity of either Rab3a or its partners might change upon stimulation. We conclude that Rab3a, together with its partners, participates in the Ca^{2+} dependence of exocytosis and that its activity is modulated further in a stimulus-dependent manner. These findings should provide some clues to elucidate the role of Rab3a in synaptic plasticity. **Key Words:** Rab3—GTP-binding protein—Exocytosis—Calcium—Chromaffin—Capacitance.
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Ca^{2+} influx through voltage-gated Ca^{2+} channels triggers the release of neurotransmitters from presynaptic terminals or the secretion of hormones from endocrine cells. The key role of Ca^{2+} ions in the release process was recognized many years ago (Douglas, 1968), but the molecular basis of this effect is still unclear (for review, see Goda and Südhof, 1997). Ca^{2+} ions not only act on the vesicles docked near the Ca^{2+} channels to cause their rapid fusion with the plasma membrane, but also at other levels to make the vesicles able to fuse, or to promote endocytosis. The exocytosis process has been dissected into a Ca^{2+}- and ATP-dependent priming step and a Ca^{2+}-dependent but ATP-independent triggering step (Bittner and Holz, 1992; Hay and Martin, 1992). The apparent affinity for Ca^{2+} ions of the priming step is in the micromolar range (Augustine and Neher, 1992; Bittner and Holz, 1992; Hsu et al., 1996), whereas higher concentrations of Ca^{2+} are required to trigger fast release of neurotransmitters or hormones (Neher and Zucker, 1993; Thomas et al., 1993; Von Rüden and Neher, 1993; Heidelberger et al., 1994; Heinemann et al., 1994). Synaptotagmin has been proposed to act as a Ca^{2+}-dependent trigger of exocytosis (Kelly, 1995; Südhof, 1995). However, there are probably other factors able to sense a rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), to convert this signal into a secretory response, and to control the magnitude of the response.

Rab3a is a monomeric GTP-binding protein belonging to the large family of Rab proteins that have been implicated in the regulation of intracellular vesicle traffic (for review, see Novick and Zerial, 1997). These proteins act as molecular switches that cycle between an active GTP-bound and an inactive GDP-bound state. Rab3a is associated with secretory vesicles (Darchen et al., 1990, 1995; Fischer von Mollard et al., 1990; Regazzi et al., 1996) and controls hormone and neurotransmitter release (Geppert et al., 1994, 1997; Holz et al., 1994; Johannes et al., 1994, 1996). Several studies strongly suggest that Rab3a exerts a negative control on the secretory response. The inhibition of Rab3a expression by antisense oligonucleotides enhanced the secretory activity of chromaffin cells during repetitive stimulation (Johannes et al., 1994). In addition, overexpression of a GTPase-deficient Rab3a protein was found to inhibit the release process in several systems (Holz et al., 1994; Johannes et al., 1994, 1996; Regazzi et al., 1996). In agreement with these findings, an increased quantal release of neurotransmitters was found in the hippocampus of mice lacking Rab3a (Geppert et al., 1997). As the size of the readily releasable pool of vesicles and the probability of Ca^{2+}-

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Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CaM, calmodulin; C_m, membrane capacitance; GAP, GTPase activating protein; NTA, nitrilotriacetic acid.

triggered release seemed unaltered, it has been suggested that Rab3a might be involved in limiting evoked release to a single quantum for each release site during a single impulse (Geppert et al., 1997; Goda and Südhof, 1997).

The mechanism of Rab3a action has not been elucidated yet. However, two putative downstream effectors of Rab3a, Rabphilin (Shirataki et al., 1993) and Rim (Wang et al., 1997), have been identified. Both of them contain C2 domains able to bind phospholipids in a Ca^{2+}-dependent manner and have been implicated in the control of the release process (Yamaguchi et al., 1993; Chung et al., 1995; McKiernan et al., 1996; Stahl et al., 1996). Therefore, Rabphilin and Rim might mediate some effects of Ca^{2+} on the release process and might provide a link between Rab3a action and the Ca^{2+} dependence of exocytosis. In agreement with this hypothesis, we observed previously that the secretory activity of chromaffin cells injected with antisense oligonucleotides directed to rab3a mRNA was increased, even at low cytosolic free $[Ca^{2+}]$, compared with that of control cells (Johannes et al., 1994). In the present study, we have used the same antisense-based strategy to investigate specifically the possible implication of Rab3a in the modulation of the Ca^{2+} dependence of exocytosis in adrenal chromaffin cells.

MATERIALS AND METHODS

Cell culture and whole-cell recordings

Bovine chromaffin cells were isolated and cultured as previously described (Darchen et al., 1990) and used 3–10 days after dissociation.

Chromaffin cells were voltage-clamped at −80 mV in the whole-cell configuration with an RK-300 patch-clamp amplifier (Biologic, France). The pipette solution contained the following (in m*M*): cesium glutamate, 145; NaCl, 4; $MgCl_2$, 2; HEPES, 10; EGTA, 10; cyclic AMP, 0.1; Na_2ATP, 2; GTP, 0.4; pH 7.25 (with CsOH). When indicated, nitrilotriacetic acid (NTA; 5 m*M*) and various amounts of $CaCl_2$ were added to this internal solution. The free $[Ca^{2+}]$ was calculated according to Föhr et al. (1993) or by software written by D. Scherman based on the constants given by Fabiato and Fabiato (1979). The recording medium contained the following (in m*M*): NaCl, 127; KCl, 5; $MgCl_2$, 2; $CaCl_2$, 5; NaH_2PO_4, 0.5; $NaHCO_3$, 5; HEPES, 10; D-glucose, 10; pH 7.25 (with NaOH). Micropipettes filled with this solution had resistances of 1–2 MΩ and made connections of 3.5 ± 2.0 MΩ. Experiments were done at room temperature (21–26°C). Capacitance was measured as described (Lledo et al., 1993), by using a dual-phase lock-in amplifier incorporated into a SWAM patch-clamp amplifier (Ljubljana, Slovenia) and by superimposing a 1,600-Hz sinusoidal voltage (1 mV peak to peak) onto the −80-mV holding potential. Where indicated (see Figs. 5 and 6), the cells were stimulated further by the application of trains of five depolarizing stimuli (from −80 mV to 0 mV, 200-ms duration, 1 Hz).

The Ca^{2+} currents were recorded using the same intrapipette solution buffered to 0.1 or 10 μ*M* free $[Ca^{2+}]$ with EGTA and NTA. As the maximal Ca^{2+} currents were obtained with depolarizing pulses to 0 mV, Ca^{2+} currents recorded 2 min after whole-cell establishment were evoked by two series of five depolarizing pulses from −80 mV to 0 mV (200-ms duration, 1 Hz). To report Ca^{2+} currents as current density, membrane capacitance (C_m) was measured by applying a hyperpolarizing pulse of 25-ms duration from −50 mV to −60 mV. The amount of charge displaced was calculated as the time integral of capacitive current, $Q = \int Idt$. Capacitance was given by the following equation: $C_m(pF) = Q(fC)/V(mV)$.

Microinjection

Antisense or control oligonucleotides (10 μg/ml) were injected in buffer containing the following (in m*M*): potassium glutamate, 135; NaCl, 20; $MgCl_2$, 4; EGTA, 0.5; GDP, 0.05; HEPES, 10, pH 7.2; and 1 mg/ml fluorescein isothiocyanate-conjugated dextran (Sigma). Just before injection, samples were cleared by centrifugation at 140,000 *g* for 15 min. Injections were performed with an Eppendorf microinjector at constant pressure. The immunofluorescent signal of coinjected fluorescein isothiocyanate-conjugated dextran allowed unambiguous identification of loaded cells. C_m measurements were made 5 days after microinjection.

Sequences were as follows: ASRab3a (5′-TGTGGCAGATGCCATCTTGTT-3′); sense (5′-AACAAGATGGCATCTGCCACA-3′); ONT3 (5′-ACTCCCAGATCTGCAGCTTGATCC-3′); ASRab3b (5′-GGTCACTGAAGCCATCTGGGA-3′). A search of the Genbank and EMBL databases did not detect any sequence that matched with ASRab3a except rab3a.

RESULTS

Effect of antisense oligonucleotides directed to rab3a on the Ca^{2+} sensitivity of exocytosis

The Ca^{2+} sensitivity of the secretory activity of adrenal chromaffin cells was studied by measuring changes in cell C_m resulting from Ca^{2+} dialysis through the patch pipette, as described by Augustine and Neher (1992). To manipulate the $[Ca^{2+}]_i$, intracellular solutions were prepared by using different Ca^{2+} buffers (EGTA or NTA). In control chromaffin cells, changes in C_m were hardly detectable at 0.1 and 0.2 μ*M* $[Ca^{2+}]_i$ and reached a maximum at 10 μ*M*. Half-maximal secretory activity occurred between 0.5 and 1 μ*M* (Figs. 1 and 2A), in agreement with previous studies (Knight and Baker, 1982; Augustine and Neher, 1992; Bittner and Holz, 1992). When the secretory activity was expressed as the maximal rate of C_m increase ($\Delta C_m/\Delta t$) and plotted against $[Ca^{2+}]_i$ (Fig. 2B), half-maximal response was obtained at 0.4–1 μ*M* $[Ca^{2+}]_i$.

We have shown previously that the secretory response to repetitive stimulation of chromaffin cells injected with antisense oligonucleotides directed to rab3a mRNA was increased compared with that of control cells (Johannes et al., 1994). To know whether Rab3a could be implicated in the Ca^{2+} sensitivity of the secretory process, we used a similar antisense-based strategy. The secretory activity of chromaffin cells microinjected with antisense oligonucleotides directed to rab3a mRNA (rab3a probe) was measured as a function of $[Ca^{2+}]_i$ and compared with the activity measured in control cells. As shown in Fig. 1, the inhibition of

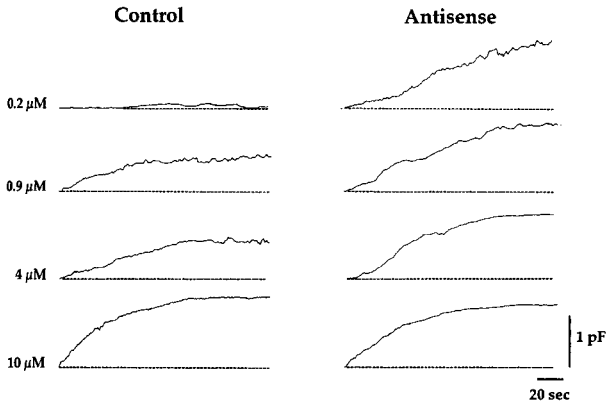

FIG. 1. C_m changes as a function of $[Ca^{2+}]_i$ in antisense-injected (Antisense, **right panel**) and noninjected (Control, **left panel**) chromaffin cells. Whole-cell recordings were performed 5 days after microinjection of an antisense oligonucleotide (ASRab3a) directed to rab3a mRNA. The patch pipette was filled with an intracellular solution containing EGTA and various amounts of Ca^{2+}. The calculated free $[Ca^{2+}]$ is indicated on the left side of the traces. The cells were kept at a holding potential of −80 mV.

Rab3a expression led to an important modification of the Ca^{2+} dependence of exocytosis. At 0.2 μM $[Ca^{2+}]_i$, i.e., a concentration that had practically no effect on exocytosis in control cells, both the net increase in C_m (Figs. 1 and 2A) and the maximal rate of C_m changes (Figs. 1 and 2B) reached near-maximal values in antisense-treated cells. Importantly, the two curves (control and antisense in Fig. 2) reach identical plateau values at 10 μM $[Ca^{2+}]_i$. Therefore, the antisense treatment resulted in a leftward shift of the Ca^{2+} dependence of the secretory activity, but did not change the maximal response. Half-maximal response was obtained at a $[Ca^{2+}]$ of 0.1–0.2 μM in antisense-treated cells instead of ~1 μM in control cells. Due to the very sharp increase in C_m observed in the antisense-treated cells when $[Ca^{2+}]_i$ was raised from 0.1 to 0.2 μM, and to the variability from cell to cell, we could not determine with a greater accuracy the $[Ca^{2+}]$ that gave half-maximal response.

Due to its high affinity for Ca^{2+} ions (K_D ~ 0.15 μM in these conditions), EGTA is appropriate to buffer $[Ca^{2+}]_i$ in the 0.1–1 μM range. However, it is less efficient at 10 μM. To confirm that the antisense treatment did not induce any significant change of the secretory activity at 10 μM $[Ca^{2+}]$, we used NTA, another Ca^{2+} chelator with a lower affinity for Ca^{2+} (K_D ~ 110 μM). As shown in Fig. 3B, when the patch pipette was filled with a solution containing a combination of EGTA, NTA, and Ca^{2+} ions corresponding to a calculated free $[Ca^{2+}]$ of 10 μM, changes in C_m were not significantly different in injected and noninjected cells.

The specificity of the rab3a probe was analyzed by monitoring the secretory activity of chromaffin cells injected with two other oligonucleotides, a "sense" oligonucleotide complementary to the rab3a probe (sense), and an antisense oligonucleotide directed to rab3b (ASRab3b). At 0.2 μM $[Ca^{2+}]_i$, the secretory activity observed in the cells injected with these control probes was similar to that measured in noninjected cells, but differed significantly from that of cells injected with the antisense rab3a probe (Fig. 3A). In contrast, at 10 μM free $[Ca^{2+}]$, no significant difference between the different conditions (rab3a probe, control probes, noninjected cells) was detected (Fig. 3B). These results, together with previous observations (Johannes et al., 1994), suggest that the effect of the rab3a probe is indeed specific.

Modulation of the activity of Rab3a upon membrane depolarization

As reported previously (Johannes et al., 1994), antisense oligonucleotides directed to rab3a induced a profound change of the secretory response elicited by a series of membrane depolarizations given at 150-s intervals. During repetitive stimulation, the response was found to decrease in control cells and to increase in

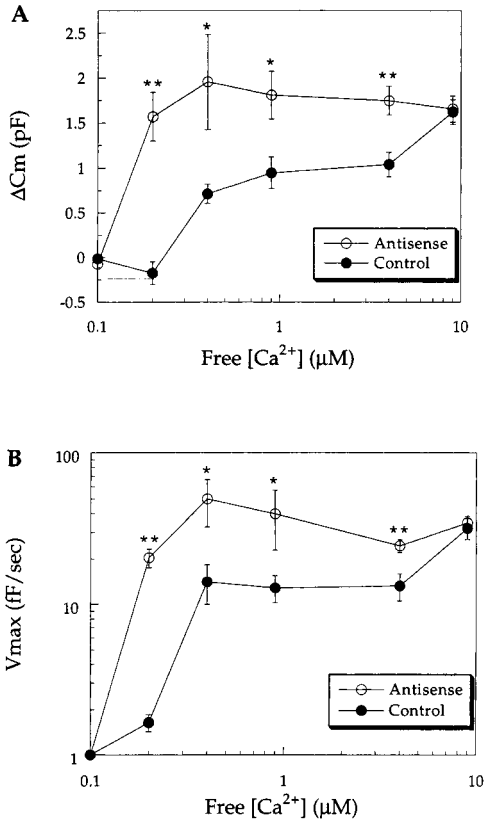

FIG. 2. Effect of the rab3a probe on the Ca^{2+} sensitivity of exocytosis. The experiments were done as described in Fig. 1. **A:** Net increase in C_m during the first 100 s of recording as a function of $[Ca^{2+}]_i$. **B:** Maximal rate of C_m increase derived from the experiments shown in A. ○, cells injected with ASRab3a antisense oligonucleotide; ●, noninjected cells. Values are given as means ± SEM; n = 4 (0.1 μM), 20 (0.2 μM), and 6–9 (0.4–10 μM). $^*p < 0.05$, $^{**}p < 0.005$ (Antisense versus Control, unpaired t test).

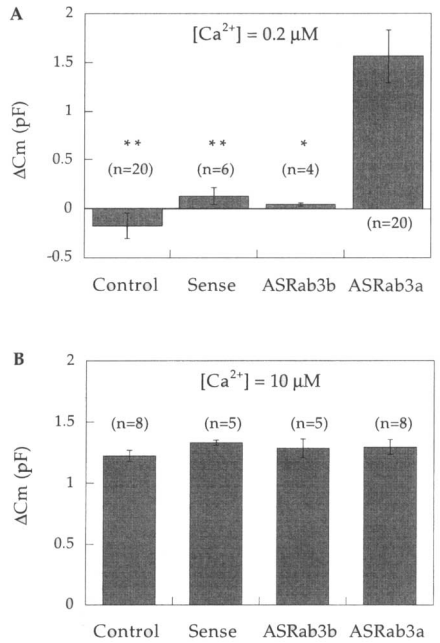

FIG. 3. Specificity of the effect of the rab3a probe. The net increase in C_m during the first 100 s after whole-cell establishment is represented (means ± SEM). Control, noninjected cells; Sense, cells injected with the sense oligonucleotide; ASRab3b, cells injected with a rab3b antisense probe; ASRab3a, cells injected with the rab3a antisense probe. **A:** The pipette solution was buffered to 0.2 μM free $[Ca^{2+}]$ with EGTA. **B:** The pipette solution was buffered to 10 μM free $[Ca^{2+}]$ with EGTA and NTA. The number of cells in each condition is shown in brackets. $^*p < 0.025$, $^{**}p < 0.005$ (versus ASRab3a, unpaired t test). In A, the values for control, sense, and ASRab3b were not statistically different.

antisense-treated cells. However, the rab3a probe had no significant effect on the magnitude of the response to the first voltage step. This might be due to the fact that $[Ca^{2+}]_i$ reached high values ($\geq$10 μM) upon membrane depolarization, because the effect of the antisense treatment was seen only at low $[Ca^{2+}]$ (i.e., $\leq$10 μM) (see Figs. 1–3). According to this view, the effect of the rab3a probe on the response to the second and to the third stimulus would suggest that either the Ca^{2+} dependence of exocytosis or the activity of Rab3a might have changed upon repetitive stimulation.

To test this possibility, we tried to analyze the effect of a train (5 × 200 ms, at 1 Hz) of membrane depolarizing stimuli on the changes in C_m elicited by dialysis with an internal solution buffered at 10 μM free $[Ca^{2+}]$. First, the Ca^{2+} currents elicited by a train of membrane depolarizations were measured at low free $[Ca^{2+}]$ (i.e., 0.1 μM) or at 10 μM free $[Ca^{2+}]$ in the pipette. The amplitude of the Ca^{2+} currents was lower at high free $[Ca^{2+}]$ than at low free $[Ca^{2+}]$ but was detected readily (Fig. 4), indicating that, under these conditions, the voltage-dependent Ca^{2+} channels were only partially inactivated. Next, Ca^{2+} currents were measured in antisense-treated cells and in control cells. The rab3a probe did not produce any significant effect on the magnitude of the currents (antisense, 31.6 ± 1.4 pA/pF; control, 30.75 ± 0.82 pA/pF; means ± SD, n = 8).

As the voltage-dependent Ca^{2+} channels were only weakly affected by high $[Ca^{2+}]$ and not by the antisense probe, it was possible to combine Ca^{2+} dialysis through the patch pipette (10 μM free $[Ca^{2+}]$) and membrane depolarizations. Before the train of stimuli (period A in Figs. 5 and 6), similar changes in C_m were observed in noninjected cells, in cells injected with either of two different antisense probes directed to rab3a (ASRab3a and ONT3), or in cells injected with either of two control probes (ASRab3b and sense). The rise in C_m that immediately followed the first train of stimuli (period B in Figs. 5 and 6) was also very similar in magnitude in antisense-treated cells and in control cells. In contrast, after the train (period C in Figs. 5 and 6), the increase in C_m was significantly higher in antisense-injected cells compared with that in control cells (a fourfold enhancement was observed). In agreement with previous results (Johannes et al., 1994), the response to a second train of membrane depolarizations was strongly increased by the antisense treatment (Fig. 5). These results thus suggest

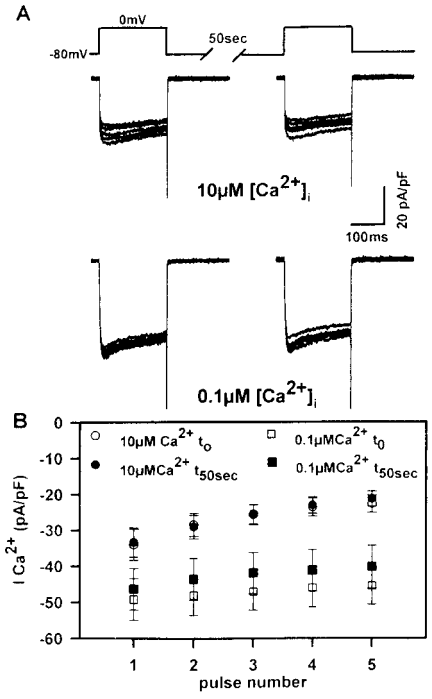

FIG. 4. Whole-cell Ca^{2+} currents recorded at different free $[Ca^{2+}]_i$. **A:** Normalized Ca^{2+} currents evoked by two series of five depolarizing pulses (from −80 mV to 0 mV) in the presence of 10 μM and 0.1 μM free $[Ca^{2+}]_i$, buffered with EGTA and NTA. **B:** Normalized Ca^{2+} current amplitude evoked by each depolarizing pulse of the first (open symbols) or the second (filled symbols) train with intrapipette solutions buffered to 10 μM (circles, n = 14) or 0.1 μM free $[Ca^{2+}]$ (squares, n = 10).

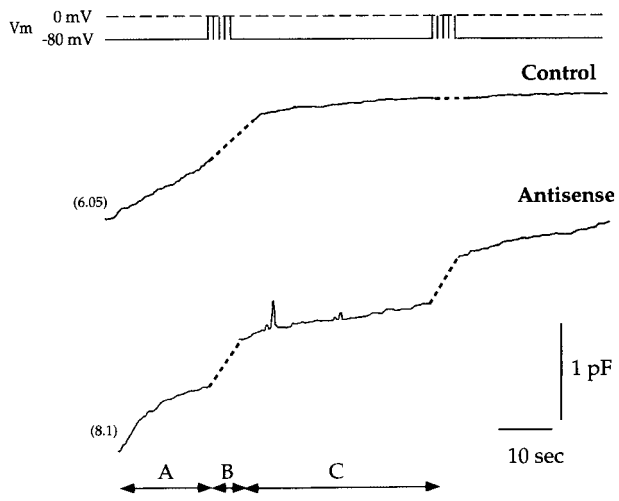

FIG. 5. Changes in C_m induced by a combination of Ca^{2+} dialysis and membrane depolarization. C_m changes were monitored in noninjected chromaffin cells (Control) or in cells injected with the rab3a probe ASRab3a (Antisense). The pipette was filled with an EGTA-containing solution buffered to 10 μM free [Ca^{2+}]. After the membrane was ruptured C_m was monitored for 20 s (period A). The cells were then challenged with a train of five depolarizing stimuli (from −80 mV to 0 mV, 200-ms duration) given at 1 Hz (indicated on the V_m trace, period B). A second train of stimuli was applied 40 s after the first one, at the end of period C. See Fig. 6 for a quantification of the results.

that the activity of Rab3a or its partners might have been modified by the electrical stimuli.

DISCUSSION

Implication of Rab3a in the Ca^{2+} dependence of exocytosis

In this study, we have provided evidence of Rab3a being implicated in the Ca^{2+} dependence of exocytosis. The inhibition of Rab3a expression by antisense oligonucleotides increased the apparent affinity of the secretory machinery for Ca^{2+} from ~1 μM to 0.1–0.2 μM without changing the maximal response obtained at 10 μM free [Ca^{2+}]. The value found in control cells agrees well with previous measurements made in permeabilized cells (Knight and Baker, 1982) and by Ca^{2+} dialysis in whole-cell recordings (Augustine and Neher, 1992) and with the affinity of the priming step (Bittner and Holz, 1992). It should be noted that fast kinetics of release of ready-to-fuse vesicles cannot be resolved by the Ca^{2+} dialysis technique, but only by membrane depolarization or photolysis of caged Ca^{2+} (Neher and Zucker, 1993; Heinemann et al., 1994; Horrigan and Bookman, 1994). Therefore, it is not possible to conclude from the data presented here whether Rab3a and its partners control the Ca^{2+} dependence of the priming or of the triggering step of exocytosis.

Our finding of a relationship between Rab3a and the Ca^{2+} dependence of exocytosis is consistent with another set of data obtained in cholinergic neurons of *Aplysia*. In these cells, a GTPase-deficient Rab3 mutant inhibits acetylcholine release. It is interesting that the Rab3-induced inhibition of release is potentiated by the intracellular injection of EGTA, which lowers $[Ca^{2+}]_i$, and reduced by conditions known to increase $[Ca^{2+}]_i$, such as trains of action potentials or paired stimuli (Doussau et al., 1998). Therefore, the apparent affinity of the release process for Ca^{2+} ions appears to be inversely related to the amount of GTP-bound Rab3a, or, in other words, the Rab3a-induced inhibition of the release process is lowered by increasing $[Ca^{2+}]_i$.

Possible mechanisms of the Ca^{2+} dependence of Rab3 action

Several Ca^{2+}-binding proteins can interact with Rab3a and therefore might account for the effect of Rab3a on the Ca^{2+} dependence of the secretory process. Rim and Rabphilin interact specifically with GTP-bound Rab3a (Shirataki et al., 1993; Wang et al., 1997), and this interaction is abolished by a mutation in the effector domain of Rab3a (McKiernan et al., 1996). Furthermore, overexpression of wild-type or truncated forms of Rim and Rabphilin modifies the secretory activity of chromaffin or PC12 cells (Chung et al., 1995; Wang et al., 1997). Therefore, it has been proposed that Rim and Rabphilin are downstream effectors of Rab3a. Both of them have C2 domains with Ca^{2+} and phospholipid binding properties. Half-maximal binding of Rabphilin to phosphatidylserine/phosphatidylethanolamine liposomes occurs at 1 μM [Ca^{2+}] (Yamaguchi et al., 1993). Rab3a might form with Rabphilin or Rim a complex that constitutes a fusion clamp. This clamp might be inactivated either by GTP hydrolysis by Rab3a or by a Ca^{2+}-induced conformational change of Rabphilin or Rim. The shift of the Ca^{2+} dependence of the secretory response reported here therefore might be a consequence of the suppression of the inhibitory complex formed by Rab3a and its downstream effectors.

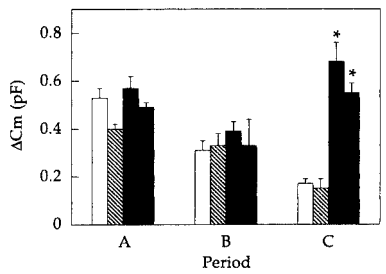

FIG. 6. Effect of a train of depolarizing stimuli on the secretory activity elicited by Ca^{2+} dialysis. C_m changes were measured in experiments made as described in Fig. 5, before (period A), during (period B), and after (period C) a train of membrane depolarizations. Noninjected cells (open columns, n = 23) or cells injected with an antisense oligonucleotide directed to rab3b or a sense probe (hatched columns, n = 11) were compared with cells injected with antisense oligonucleotides directed to rab3a (ASRab3a, black columns, n = 21, or ONT3, gray columns, n = 4). *$p < 0.001$, unpaired t test.

We and others have shown that GTP hydrolysis by Rab3a is rate-limiting in the exocytotic process in chromaffin cells and in cholinergic neurons of *Aplysia* (Holz et al., 1994; Johannes et al., 1994, 1996). In addition, it has been reported that GTP hydrolysis by Rab3a occurs upon stimulation of synaptosomes (Stahl et al., 1994). Therefore, another possibility that could fit with the data presented here is that GTP hydrolysis by Rab3a is triggered by a rise in $[Ca^{2+}]_i$. Increasing $[Ca^{2+}]$ would gradually switch Rab3a into the "off" state, leading to enhanced secretory response. The low intrinsic GTPase activity of Rab3a can be increased by the recently identified Rab3-GAP (GTPase activating protein) (Fukui et al., 1997). It will thus be interesting to test whether Rab3-GAP activity is increased by Ca^{2+}, either directly or via activation by a protein kinase.

Calmodulin (CaM) is another Ca^{2+}-binding protein that might be involved in the functional link between Rab3a and the Ca^{2+} dependence of exocytosis. Park et al. (1997) have reported recently that Ca^{2+}/CaM interacts with Rab3a and causes it to dissociate from synaptic membranes. In contrast to Rab guanine nucleotide dissociation inhibitor, which can extract GDP- but not GTP-bound Rab3a from synaptic vesicle membranes, Ca^{2+}/CaM acts on Rab3a either bound to GDP or to GTP. It is interesting that the Ca^{2+} dependence of the CaM-induced dissociation of Rab3a is strikingly similar to the Ca^{2+} dependence of exocytosis, and to the range of $[Ca^{2+}]$ where an effect of the rab3a probe is observed (see Figs. 1 and 2). Indeed, half-maximal and maximal effects of CaM on Rab3a were observed at 0.5 and 10 μM $[Ca^{2+}]$, respectively. Therefore, Ca^{2+} ions could inactivate Rab3a via CaM-induced dissociation of Rab3a from the membrane of secretory vesicles.

A role for Rab3a in synaptic plasticity

Our data also suggest that the effect of Rab3a on the Ca^{2+} dependence of exocytosis might be modulated further in a stimulus-dependent manner. By combining Ca^{2+} dialysis through the patch pipette and membrane depolarization, we observed (a) the absence of any effect of the rab3a probe on the secretory activity at 10 μM free $[Ca^{2+}]$ before and during the depolarizing stimuli and (b) a large increase in C_m changes after the train of stimuli in antisense-injected cells compared with that in control cells. Therefore, these results indicate that a train of membrane depolarizations induced a difference between antisense-treated cells and control cells with respect to their ability to secrete in response to 10 μM free $[Ca^{2+}]$.

One might propose two possibilities that could account for this observation. First, Rab3a might control the refilling of the pool of releasable vesicles. In the presence of normal amounts of Rab3a, the refilling step would be limiting, leading to rapid exhaustion of this pool of vesicles under repeated stimulation. However, in the absence of superimposed membrane depolarization, the kinetics of C_m increase and the plateau value of C_m at 10 μM free $[Ca^{2+}]$ are very similar in antisense-treated cells and in control cells. Thus, it seems unlikely that the difference observed after a train of stimuli might be due to a lower size of the pool of releasable vesicles in control cells compared with that in antisense-treated cells. The second possibility is that membrane depolarizations increase the ability of Rab3a to inhibit exocytosis or make the "Rab3a clamp" more difficult to remove. Future work will be needed to investigate whether Ca^{2+}-dependent activation of protein kinases might change the properties of downstream effectors of Rab3, such as Rabphilin and Rim or the activity of Rab3-GAP. It is already known that Rabphilin is phosphorylated by protein kinase A or by CaM/kinase II (Kato et al., 1994; Numata et al., 1994; Fykse et al., 1995), but the physiological meaning of this modification is not known. It should be noted that capacitance measurements reflect the sum of two activities: exocytosis and endocytosis. However, it seems unlikely that an effect of the rab3a antisense probe on endocytosis could account for the higher rate of C_m increase observed after the train of membrane depolarizations in antisense-injected cells without having any effect before the train. In addition, the lack of response to the second train of stimuli observed in control cells, in contrast to that in antisense-injected cells, also argues against an effect on endocytosis.

Rab3a has been implicated in several forms of synaptic plasticity. The knockout of rab3a gene in mice results in an increase of paired-pulse facilitation in the CA3 region of the hippocampus (Geppert et al., 1997). Paired-pulse facilitation is also increased in cholinergic neurons of the buccal ganglion of *Aplysia* after microinjection of a GTPase-deficient Rab3 mutated protein (Doussau et al., 1998). Moreover, the implication of Rab3 in synaptic plasticity is not restricted to short-term phenomena, because Castillo et al. (1997) have reported the absence of long-term potentiation in hippocampal mossy fibers of mice lacking Rab3a. It seems likely that the implication of Rab3 in the Ca^{2+} dependence of exocytosis reported here and by Doussau et al. (1998) could underlie some aspects of synaptic plasticity.

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