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Regulation of Releasable Vesicle Pool Sizes by Protein Kinase A-Dependent Phosphorylation of SNAP-25

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Summary

Protein kinase A (PKA) is a key regulator of neurosecretion, but the molecular targets remain elusive. We combined pharmacological manipulations of kinase and phosphatase activities with mutational studies on the exocytotic machinery driving fusion of catecholamine-containing vesicles from chromaffin cells. We found that constitutive PKA activity was necessary to maintain a large number of vesicles in the release-ready, so-called primed, state, whereas calcineurin (protein phosphatase 2B) activity antagonized this effect. Overexpression of the SNARE protein SNAP-25a mutated in a PKA phosphorylation site (Thr-138) eliminated the effect of PKA inhibitors on the vesicle priming process. Another, unidentified, PKA target regulated the relative size of two different primed vesicle pools that are distinguished by their release kinetics. Overexpression of the SNAP-25b isoform increased the size of both primed vesicle pools by a factor of two, and mutations in the conserved Thr-138 site had similar effects as in the a isoform.

Introduction

Activity-dependent regulation of protein kinases and phosphatases is responsible for the remarkable ability of neurons and neurosecretory cells to modulate their secretory/synaptic strengths in accordance with changing physiological requirements. Presynaptic cAMP-dependent protein kinase (PKA) activation facilitates release in neurons (Capogna et al., 1995; Trudeau et al., 1996) and forms the basis for a form of LTP (Weisskopf et al., 1994). Inhibition of the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin (protein phosphatase 2B) leads to increased neurotransmitter release (Antoni et al., 1993; Nichols et al., 1994; Sihra et al., 1995; Victor et al., 1995; Sistiaga and Sánchez-Prieto, 2000), indicating a negative role of calcineurin in the regulation of vesicle release. In rat pancreatic α cells, calcineurin counteracted the effect of a forskolin-induced increase in cAMP on vesicle priming (Gromada et al., 2001). These data indicate that a balance between PKA and calcineurin activity may control the exocytotic strength.

Published data on the effect of cAMP on secretion of catecholamines from chromaffin cells are heterogeneous: in some studies no effect or an inhibition was found (Baker et al., 1985; Cheek and Burgoyne, 1987), whereas in most cases a stimulatory effect was identified, which, however, varied widely in magnitude between studies (Bittner et al., 1986; Morita et al., 1987; Malhotra et al., 1989; Parramón et al., 1995; Przywara et al., 1996; Warashina, 1998). The effect of cAMP in intact cells involves depolarization of the membrane potential, Ca^{2+} influx, and an increase in the basal $[Ca^{2+}]_i$ of several hundred nM (Parramón et al., 1995; Przywara et al., 1996). Calcium has several effects on exocytosis: it increases the recruitment of large dense-core vesicles into the releasable vesicle pools by both PKC-dependent and PKC-independent mechanisms (Smith et al., 1998), and in addition, two calcium sensors are involved in executing release (Voets, 2000). On the other hand, cAMP production can be induced in chromaffin cells by increases in basal $[Ca^{2+}]_i$ (Keogh and Marley, 1991), indicating that the effect of raised basal $[Ca^{2+}]_i$ and exogeneous cAMP may occlude each other. Thus, it is not hard to see how conflicting results about the role of cAMP can be obtained by differences in cell preparation and stimulation regimes, which may, for instance, affect resting $[Ca^{2+}]_i$. The use of permeabilized chromaffin cells identified the catalytic subunit of PKA as one of several stimulatory factors that leak from the cells, causing run-down of secretion (Morgan et al., 1993). Since in this preparation the effect of PKA on $[Ca^{2+}]_i$ is bypassed, it was concluded that the stimulatory effect must be on the exocytotic machinery itself (Morgan et al., 1993). However, in this study the question remained open whether cAMP regulates the availability of release-ready vesicles or the release process itself.

Several presynaptic proteins serve as substrates for PKA-dependent phosphorylation: α-SNAP (Hirling and Scheller, 1996), cysteine string protein (Evans et al., 2001), synapsin I (Jovanovic et al., 2001), snapin (Chheda et al., 2001), RIM1 (Lonart et al., 2003), and SNAP-25 (Risinger and Bennett, 1999; Hepp et al., 2002). The recent demonstration that phosphorylation of RIM1α by PKA is necessary for induction of presynaptic LTP at cerebellar parallel fiber synapses (Lonart et al., 2003) raises the interesting possibility that synaptic/secretory strength may be controlled by a small number of key PKA targets. However, overexpression of a RIM1α serine-to-aspartate mutation (S413D) in an attempt to mimic the phosphorylated state did not suffice to increase EPSC amplitude, leaving the question open whether phosphorylation of RIM1α is sufficient to induce LTP in this system (Lonart et al., 2003). Nevertheless, the definition of a minimum set of targets required to induce synaptic/secretory changes now seems within reach.

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SNAP-25 (synaptosome-associated protein of 25 kDa) is a component of the SNARE complex, which is formed between the plasma membrane and the vesicle in preparation for exocytosis. The SNARE complex consists of a twisted bundle of four α helices, two from SNAP-25 (t-SNARE) and one each from syntaxin (t-SNARE) and synaptobrevin (v-SNARE) (Sutton et al., 1998). Syntaxin and synaptobrevin are anchored in the plasma membrane and the vesicle membrane, respectively, via transmembrane domains, whereas SNAP-25 is bound to the plasma membrane by palmitoylation of four cysteine residues in the linker region between the two α helices (Hess et al., 1992). The SNARE complex may be preassembled during priming of the vesicle for release (Xu et al., 1998, 1999; Chen et al., 2001), whereas the final "zipping-up" of the complex toward the membrane anchors of syntaxin and synaptobrevin may trigger fusion (Hanson et al., 1997). Thus, priming and depriming (the reverse process of priming) may coincide with (or even be driven by) initial formation and dissociation of the SNARE complex, respectively. Since the number of release-ready vesicles will be determined by the relationship between priming and depriming rates, the SNARE complex may act as a regulator of the number of release-ready vesicles.

In the present study, we have investigated the regulation of the two releasable (primed) vesicle pools in chromaffin cells, the slowly releasable pool (SRP) and the readily releasable pool (RRP), by PKA and phosphatases. We found that PKA and calcineurin have antagonistic effects on the size of the SRP and the RRP. Overexpression of SNAP-25 T138D and SNAP-25 T138A, mutants modified in the PKA phosphorylation site, mirrored the effects of PKA activation and inhibition, respectively, and eliminated the effect of PKA inhibitors on the SRP, but not on the RRP. Consequently, phosphorylation of SNAP-25 regulates the size of the SRP. Due to the linear arrangement of the SRP and RRP pools (Figure 7D), this leads to an indirect effect of SNAP-25 phosphorylation on the RRP. In addition, one more PKA target regulates RRP size.

Results

PKA Activity Is Constitutively Stimulatory for Secretion

In our investigation, we wanted to study the effect of cAMP-dependent phosphorylation on the exocytotic machinery itself. We therefore bypassed the effects of cAMP on $[Ca^{2+}]_i$ (see Introduction) by performing whole-cell patch clamp in the presence of a photolysable calcium chelator nitrophenyl-EGTA (NP-EGTA) and a combination of two calcium-sensitive dyes, which allowed us to accurately control and monitor basal $[Ca^{2+}]_i$ online during the experiment (Voets, 2000). In different experimental series, the basal $[Ca^{2+}]_i$ was elevated to similar levels by slow photolysis of NP-EGTA before stimulation (Voets, 2000). Thus, any effects that cAMP might have on the basal $[Ca^{2+}]_i$ was cancelled. The basal $[Ca^{2+}]_i$ during our studies was adjusted to a rather high level (several hundred nM; shown in each figure) for two reasons. First, the K_D of NP-EGTA is relatively high (80 nM; Ellis-Davies and Kaplan, 1994), so that a basal $[Ca^{2+}]_i$ of several hundred nM is required to saturate the chelator and allow flash photolysis experiments. Second, a high basal $[Ca^{2+}]_i$ induces maximal vesicle priming, allowing an accurate determination of the size of releasable vesicle pools (Voets, 2000).

To monitor catecholamine secretion from chromaffin cells, we combined capacitance and amperometric measurements (Rettig and Neher, 2002). Cells were held in whole-cell configuration, and flash photolysis of NP-EGTA was used to induce a step-like, homogenous increase of $[Ca^{2+}]_i$ from 400–600 nM to 10–30 μM. This stimulus evoked an increase in membrane capacitance and the released catecholamines were simultaneously detected by a carbon fiber pushed gently toward the cell surface (Figure 1A). Secretion typically consists of a rapid burst phase, corresponding to the complete emptying of the releasable vesicle pools, followed by a slower sustained phase representing recruitment of vesicles and consecutive exocytosis (Ashery et al., 2000). Detailed kinetic analysis of the capacitance response revealed that the burst phase itself consists of two components, the fast (time constant, τ_1 = 20–30 ms) and the slow (τ_2 = 200–300 ms) burst component (Xu et al., 1998; Voets, 2000). These components represent the parallel fusion of two release competent vesicle pools, the readily (RRP) and the slowly (SRP) releasable pool, respectively (our current kinetic model is seen in Figure 7D).

Inclusion of PKA activators into the pipette solution (100 μM cAMP and 50 μM forskolin; ~3 min was allowed for the compounds to diffuse into the cells; Pusch and Neher, 1988) did not change flash-evoked exocytosis (Figure 1A, left panel). A second flash stimulation was given 80–100 s after the first one, to test for refilling of the releasable vesicle pools. Previously, we have shown that inhibition of PKC does not influence the first flash stimulation; however, the response to a second stimulation is reduced (Nagy et al., 2002), indicating that PKC activity is required for refilling of the vesicle pools within this time interval. In the present study, the second stimulation evoked a response similar to the first one and this was unaffected by cAMP (Figure 1A, right panel). When we perfused the cells through the patch pipette with a mixture of PKA inhibitors (1 μM KT5720 and the PKA pseudosubstrate 1 μM PKI 6–22 peptide for 3 min), a reduction of the exocytotic burst was observed (Figure 1B). Kinetic analysis was performed by fitting a sum of exponential functions to the capacitance traces, allowing a determination of pool sizes and fusion kinetics for the SRP and the RRP, and the rate of sustained release (see Experimental Procedures). This analysis showed that both the SRP and the RRP sizes were reduced ~50% after PKA inhibition, while the sustained component remained unaffected (Figure 6A). The fusion kinetics from the RRP and SRP were unchanged in all cases (PKA activation: $\tau_1 = 16 \pm 2$ ms, $\tau_2 = 249 \pm 37$ ms; corresponding control cells: $\tau_1 = 22 \pm 3$ ms, $\tau_2 = 213 \pm 56$ ms; PKA inhibition: $\tau_1 = 26 \pm 5$ ms, $\tau_2 = 191 \pm 31$ ms; corresponding control cells: $\tau_1 = 21 \pm 3$ ms, $\tau_2 = 239 \pm 40$ ms), indicating that PKA does not regulate the fusion triggering step itself. The response to a second flash stimulation mirrored that of the first one (Figure 1B, right panel). Taken together, we conclude that under our

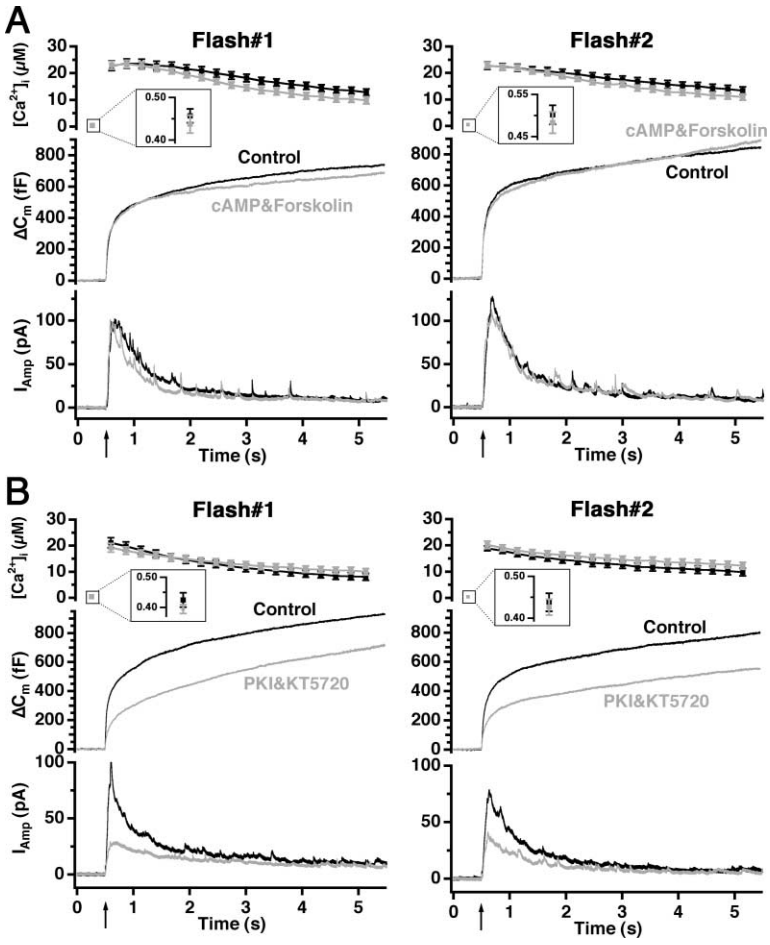

Figure 1. Inhibition of PKA Reduces the Exocytotic Burst

(A) Mean $[Ca^{2+}]_i$ (upper panel; error bars represent SEM), capacitance change (middle panel), and amperometric current (lower panel) after a step-like elevation of $[Ca^{2+}]_i$ caused by flash photolysis of caged calcium (flash at arrow). Both capacitance and amperometric traces display a rapid, burst-like increase within the first 0.5 s after the flash, followed by a slower sustained phase of secretion. Infusion of PKA activators (100 μM cAMP and 50 μM forskolin) into the cell for 3 min (gray traces; n = 17 cells) did not change secretion compared to nontreated cells (black traces; n = 17). The secretion evoked by a second flash stimulation (80–100 s after the first one; right panel) was also unaffected. The inset in the upper panel shows the basal $[Ca^{2+}]_i$ before the flash that was not significantly different between the two groups.

(B) Cells infused with PKA inhibitors (1 μM PKI 6–22 and 1 μM KT5720 for 3 min) had a reduced exocytotic burst (gray traces; n = 20) compared to the control group (black traces; n = 21). A similar reduction in the burst phase was observed after the second flash stimulation (right panel).

recording conditions, PKA has a constitutive stimulatory effect on the size of the releasable vesicle pools.

The Effect of PKA Is Antagonized by Calcineurin

We next tested whether inhibition of phosphatases would modify secretion. Figure 2A shows that preincubation of cells with the calcineurin inhibitor cypermethrin (100 nM in the bath for at least 10 min before establishing the whole-cell configuration) did not affect flash-evoked secretion. Also, the response to a second flash stimulation was unaffected (Figure 2A, right panel). In agreement with previous studies (Gutierrez et al., 1995; Gil et al., 2000), we observed that cells preincubated with 100 nM calyculin A (an inhibitor of protein phosphatases 1 and 2A) longer than 10 min underwent morphological changes. Therefore, we reduced preincubation time to ~5 min, which did not affect secretion evoked by the first flash (left panel in Figure 2B), but potentiated the second flash-evoked response (right panel in Figure 2B).

Next, we combined preincubation with phosphatase inhibitors and infusion of PKA inhibitors. Figure 2C shows that inhibition of calcineurin by cypermethrin completely prevented the effect of PKA inhibitors on secretion, whereas calyculin A was ineffective (Figures 2D and 6A). The result of a second stimulation mirrored that of the first (not shown). Note that the simultaneously measured amperometric signal confirms that the effect of cypermethrin was due to rescue of exocytosis from PKA inhibition and was not caused by a difference in endocytosis. These data show that calcineurin antagonizes the effect of PKA, and thus the relative activity of the two enzymes appears to determine the size of the releasable vesicle pools. This is similar to the situation in pancreatic α cells (Gromada et al., 2001), except that in our case this balance is shifted in favor of PKA.

Protein Kinase A Phosphorylates SNAP-25 in Chromaffin Cells

In a search for the targets of PKA, we tested whether PKA phosphorylates SNAP-25 in chromaffin cells. Previous experiments showed that SNAP-25 is phosphorylated in vitro and the phosphorylation site was mapped to Thr-138 (Figure 3A; Risinger and Bennett, 1999). We raised an antibody against a synthetic phosphopeptide containing residues 132–143 of SNAP-25 including phosphorylated threonine at position 138 (Experimental Procedures). After purification, we tested the specificity in HEK cells overexpressing either native SNAP-25a or the T138A mutant. Figure 3B shows that the phosphopeptide-specific antibody detected a protein of approximately 25 kDa in SNAP-25a-overexpressing cells that were treated with PKA activators (ninth lane). Untreated SNAP-25a-overexpressing cells provided only a faint band (third lane), which was reduced by PKA inhibition

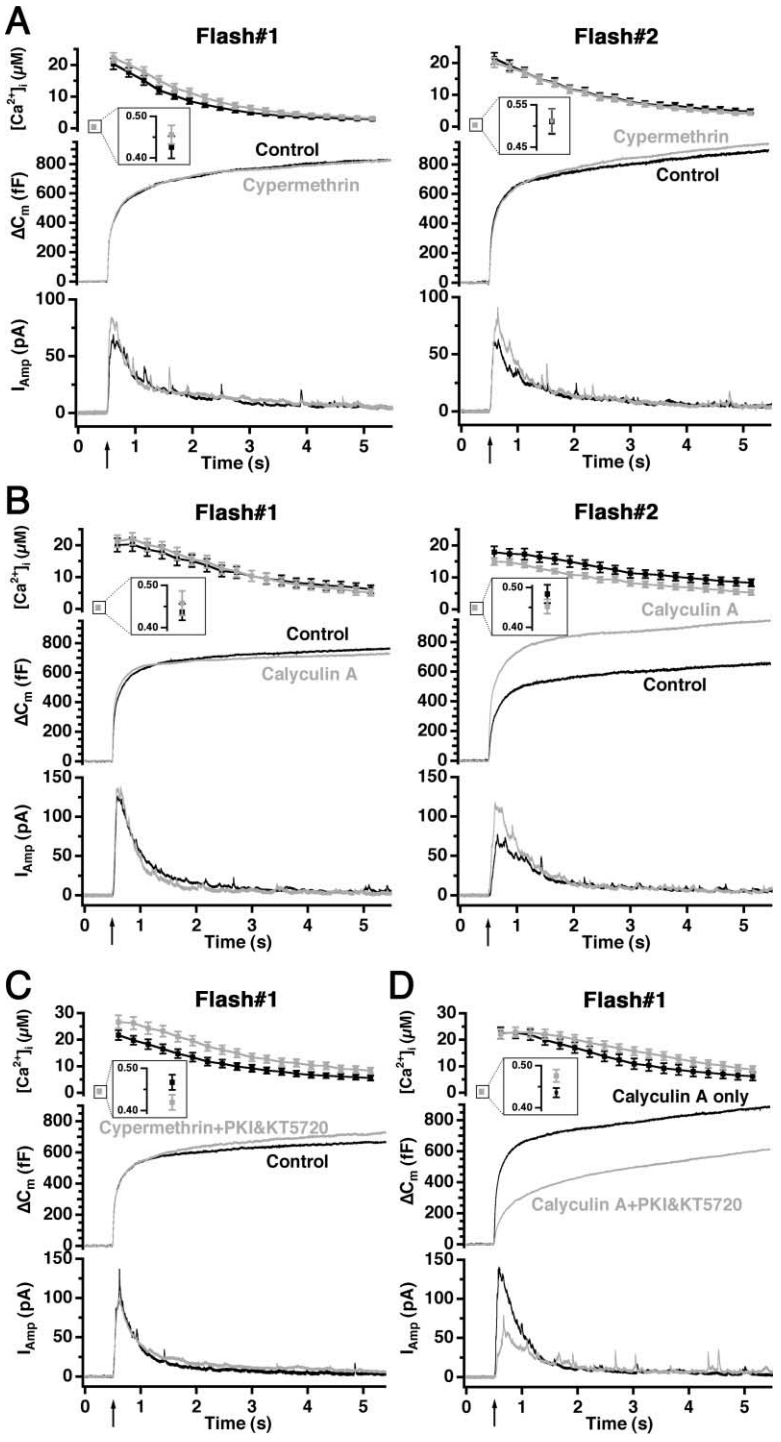

Figure 2. Inhibition of Calcineurin but not Phosphatase 1 and 2A Prevents the Effect of PKA Inhibition

(A) The mean $[Ca^{2+}]_i$, capacitance change, and amperometric response to the first flash stimulation (for explanations see legend to Figure 1). Preincubation of chromaffin cells with the calcineurin inhibitor cypermethrin (100 nM for at least 10 min) did not change flash-evoked catecholamine secretion (gray traces; n = 22) as compared to nontreated cells (black traces; n = 20). Left panel: first flash-evoked response; right panel: second flash-evoked response.

(B) Left panel: preincubation with the type 1 and 2A phosphatase inhibitor calyculin A (100 nM for 5–10 min; gray traces, n = 18) had no effect on the first flash-evoked response (black traces: untreated cells, n = 19). Right panel: calyculin A increased the response to the second flash stimulation.

(C) Cells were preincubated with cypermethrin (100 nM for 10 min) and infused with PKA inhibitors (1 μM PKI 6–22 and 1 μM KT5720 for 3 min) (gray traces; n = 17). Inhibition of calcineurin prevented the effect of PKA inhibition, leading to a normal secretory response as compared to control cells not treated with cypermethrin and PKA inhibitors (black traces; n = 18).

(D) Inhibition of protein phosphatase 1 and 2A with calyculin A (100 nM for 5 min) did not prevent the detrimental effect of PKA inhibition (3 min intracellular perfusion with 1 μM PKI 6–22 and 1 μM KT5720). Thus, inhibition of PKA led to a similar reduction of the exocytotic burst (gray traces; n = 16) than without calyculin A (for comparison see Figure 1B). Black traces display results from cells treated with calyculin but not PKA inhibitors (n = 16).

(sixth lane). The band was absent in lysates of cells overexpressing T138A mutant (second, fifth, and eight lanes) or in lysates of nontransfected cells (first, fourth, and seventh lanes).

Alternative splicing of exon 5 gives rise to two SNAP-25 splice variants, SNAP-25a and SNAP-25b, which differ by 9 amino acid substitutions (Bark and Wilson, 1994). The isoforms are differentially expressed: the a isoform is present in neurosecretory cells (including chromaffin cells) and the embryonic brain, whereas the b isoform dominates in adult brain (Bark et al., 1995). The alternatively spliced exon 5 encodes amino acids 56–94, so that the PKA phosphorylation site at T138 and flanking regions are conserved between isoforms. It was therefore no surprise that PKA also phosphorylates the SNAP-25b isoform (data not shown).

We next tested whether PKA phosphorylates endogeneous SNAP-25 in chromaffin cells (Figure 3C). A weak band was observed with the phosphopeptide-specific antibody in immunoblots of control cells (first lane),

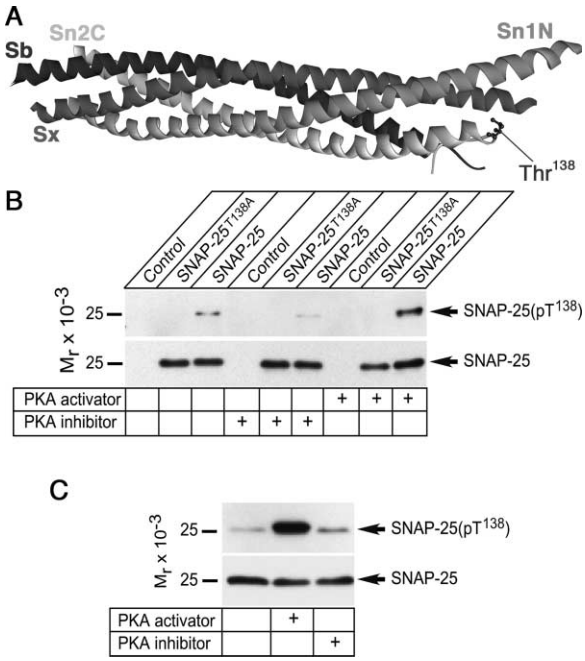

Figure 3. PKA Phosphorylates SNAP-25 at Thr-138 in Chromaffin Cells

(A) The position of SNAP-25 Thr-138 in the SNARE complex. Sb is synaptobrevin, Sx is syntaxin, Sn1N is the N-terminal α helix of SNAP-25, and Sn2C is the C-terminal helix of SNAP-25 (modified after Sutton et al., 1998). Membrane anchors would attach at the left end of the complex.

(B) HEK cell homogenates were subjected to SDS-PAGE and transferred on nitrocellulose membranes. Membranes were probed with SNAP-25 antibodies detecting either total SNAP-25 (mAb 71.1, lower panels marked SNAP-25) or with the phosphopeptide-specific antibody (upper panels marked SNAP-25(pT^{138})). Increased phosphorylation of SNAP-25 was detectable on PKA activation (20 min of incubation with 50 μM forskolin, 100 μM IBMX, and 1 mM 8-Br-cAMP) if cells were transfected with native SNAP-25 (upper panel, ninth lane), whereas a weak signal for phosphorylated SNAP-25 was obtained without PKA activation (third lane). The amount of phospho-SNAP-25 was further reduced if PKA was inhibited (1 μM KT5720; sixth lane). Immunoblot analysis of homogenates of untransfected HEK cells (first, fourth, and seventh lanes) and cells transfected with SNAP-25 T138A (second, fifth, and eighth lanes) did not give any signal with the phosphopeptide-specific antibody.

(C) A strong signal was detected at 25 kDa with the phosphopeptide-specific antibody (SNAP-25(pT^{138})) after activation of PKA in bovine chromaffin cells (upper panel, second lane), whereas only a weak band was visible in control cells (upper panel, first lane) and cells treated with PKA inhibitors (upper panel, third lane).

as well as in cells treated with PKA inhibitor (third lane). However, activation of PKA led to the detection of a strong signal (second lane). These data show that SNAP-25 can be phosphorylated in chromaffin cells at T138, in agreement with recent data from PC-12 cells (Hepp et al., 2002).

Mutation of the PKA Phosphorylation Site in SNAP-25

We next asked whether the phosphorylation of SNAP-25 contributes to the constitutive stimulatory effect of PKA on exocytosis. In order to answer this question, we replaced T138 in the SNAP-25a isoform either by the negatively charged aspartate (T138D), creating a mutant, which we speculated may be phosphomimetic, or by alanine (T138A), which cannot be phosphorylated. We overexpressed these mutants coupled with Green Fluorescent Protein (GFP) as an expression marker using the Semliki Forest Virus system (SFV) (Ashery et al., 1999). Previously, we have shown that overexpression of the GFP-SNAP-25a fusion protein leads to a ~25-fold overexpression over the native SNAP-25a and that the fusion protein assembles into SNARE complexes in the living cell (Wei et al., 2000) without changing flash-induced exocytosis of the infected cells (Nagy et al., 2002). Thus, a basic assumption of our approach is that the expressed GFP-linked SNAP-25a is functional and substitutes for the native SNAP-25 in the cell. This has been confirmed by the rescue of secretion in chromaffin cells from SNAP-25 knockout mice upon overexpression of SNAP-25a (Sørensen et al., 2003) or GFP-linked SNAP-25a (G.N., unpublished) and by the observation that overexpression of mutated SNAP-25 displayed a dominant phenotype when overexpressed in bovine cells (Wei et al., 2000; Sørensen et al., 2002; Nagy et al., 2002).

Figure 4A shows that overexpression of the phosphomimetic T138D mutant resulted in a moderate but not significant (see Figure 6) increase in the sustained phase of secrection. The characteristics of the second flash-evoked secretion were also not altered. However, when overexpressing the T138A mutant, the exocytotic burst component was reduced similarly to PKA inhibition (Figure 4B, compare Figure 1B). Kinetic analysis revealed that the T138A mutant reduced the size of both the RRP and the SRP by ~60% without changing the rate of the sustained component (Figure 6). The fusion kinetics of SRP and RRP release processes were unaffected (cells overexpressing SNAP-25a T138D: $\tau_1 = 24 \pm 5$ ms, $\tau_2 = 203 \pm 42$ ms; corresponding control cells: $\tau_1 = 21 \pm 2$ ms, $\tau_2 = 278 \pm 41$ ms; cells overexpressing SNAP-25a T138A: $\tau_1 = 28 \pm 6$ ms, $\tau_2 = 180 \pm 40$ ms; corresponding control cells: $\tau_1 = 20 \pm 3$ ms, $\tau_2 = 198 \pm 27$ ms).

Using chromaffin cells from a SNAP-25 knockout mouse, we recently showed that the two SNAP-25 splice variants differentially regulate the size of the exocytotic burst (Sørensen et al., 2003). Overexpression of SNAP-25a in SNAP-25 knockout or wild-type mouse cells resulted in similar secretion as in wild-type cells, whereas overexpression of SNAP-25b induced a 2- to 3-fold larger burst size. In the next set of experiments, we asked whether the effects of phosphorylation and isoform are independent of each other.

As was the case in mouse cells, overexpression of the SNAP-25b isoform in bovine chromaffin cells increased cathecholamine secretion (Figure 5A). Note that in this series of measurements, secretion from control cells was lower than in previous measurement series; this illustrates the importance of making control experiments from the same cell preparations. Kinetic analysis revealed a ~2-fold increase in the size of both burst components, whereas the sustained component was not significantly altered (Figure 5B). The fusion kinetics of the SRP and the RRP were unaffected (cells overexpressing SNAP-25b: $\tau_1 = 23.5 \pm 2.5$ ms, $\tau_2 = 255 \pm 30$ ms; control cells: $\tau_1 = 24 \pm 4$ ms, $\tau_2 = 261 \pm 26$ ms). The second flash also evoked an increased exocytotic burst in cells overexpressing SNAP-25b (data not

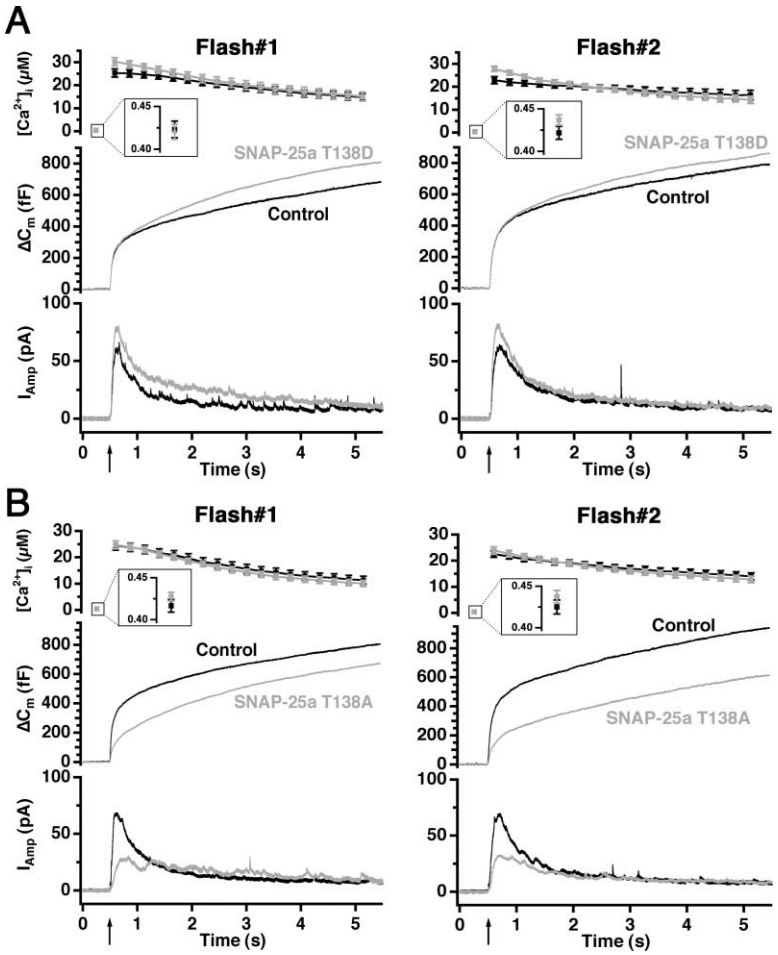

Figure 4. The SNAP-25a T138A Mutant Reduces the Exocytotic Burst Size

(A) Overexpression of the SNAP-25a T138D mutant slightly and nonsignificantly (see Figure 6) increased the sustained component of flash-evoked secretion (gray traces; n = 23). There was no systematic difference between the first (left panel) and second (right panel) flash-evoked response (noninfected control cells are displayed with black; n = 22).

(B) Overexpression of the T138A mutant reduced the exocytotic burst (gray traces; n = 25) without changing the sustained phase compared to noninfected control cells (black traces; n = 22). A similar reduction was observed for the second flash response (right panel).

shown). Thus, together with the previous finding that overexpression of wild-type SNAP-25a does not change secretion in bovine chromaffin cells (Nagy et al., 2002), we can conclude that SNAP-25a is the major functional isoform in bovine as well as in mouse chromaffin cells.

We introduced the phosphorylation mutations T138D and T138A into the SNAP-25b isoform and compared secretion from cells expressing this mutant to that obtained after overexpression of wild-type SNAP-25b. Figure 5C shows that overexpression of the T138D mutant resulted in a similar phenotype as wild-type SNAP-25b, whereas overexpression of the T138A mutant led to a reduced burst size compared to wild-type SNAP-25b (Figure 5D). Kinetic analysis revealed that the T138A mutation reduced both burst components by ~60% compared to SNAP-25b wild-type, the same percentage reduction as seen when the T138A mutation was introduced into SNAP-25a (Figure 6B). The sustained component and the fusion kinetics of SRP and RRP fusion were not significantly changed by the mutations (cells overexpressing SNAP-25b T138D: $\tau_1 = 24 \pm 2.5$ ms, $\tau_2 = 280 \pm 41$ ms; corresponding cells overexpressing wild-type SNAP-25b: $\tau_1 = 30 \pm 3$ ms, $\tau_2 = 208 \pm 25.5$ ms; cells overexpressing SNAP-25b T138A: $\tau_1 = 25 \pm 3$ ms, $\tau_2 = 201 \pm 28$ ms; corresponding cells overexpressing wild-type SNAP-25b: $\tau_1 = 23.5 \pm 3$ ms, $\tau_2 = 195 \pm 17$ ms).

In conclusion, both the choice of SNAP-25 isoform and the mutation of T138 change the size of the SRP and RRP vesicle pools by a factor of ~2; SNAP-25b causes 2-fold larger pool sizes than SNAP-25a, and T138A causes 2-fold lower pool sizes than T138D (or nonexpressing cells). When combined with each other, these effects appear independent of each other; thus, overexpression of SNAP-25b T138A induces the same level of exocytosis as in nonexpressing cells (where secretion is driven by SNAP-25a, compare Figures 5A and 5D).

Phosphorylation of SNAP-25 Is Involved in the Effect of PKA on Secretion

The similarity between secretion in the presence of PKA inhibitors (Figure 1B) and when overexpressing SNAP-25a T138A (Figure 4B) suggests that phosphorylation of SNAP-25 may be involved in the constitutive stimulatory effect of PKA. In order to address this question directly, we infused PKA inhibitors into cells overexpressing the T138D mutant of SNAP-25a and compared it to T138D-expressing cells without inhibitor. As seen in Figure 7A, inhibition of PKA led only to a slight reduction in secretion under these conditions compared to the effect on uninfected cells (Figure 1B). When we combined overexpression of SNAP-25a T138A with application of PKA inhibitors, we observed a similar slight reduction compared to overexpression of SNAP-25a T138A without

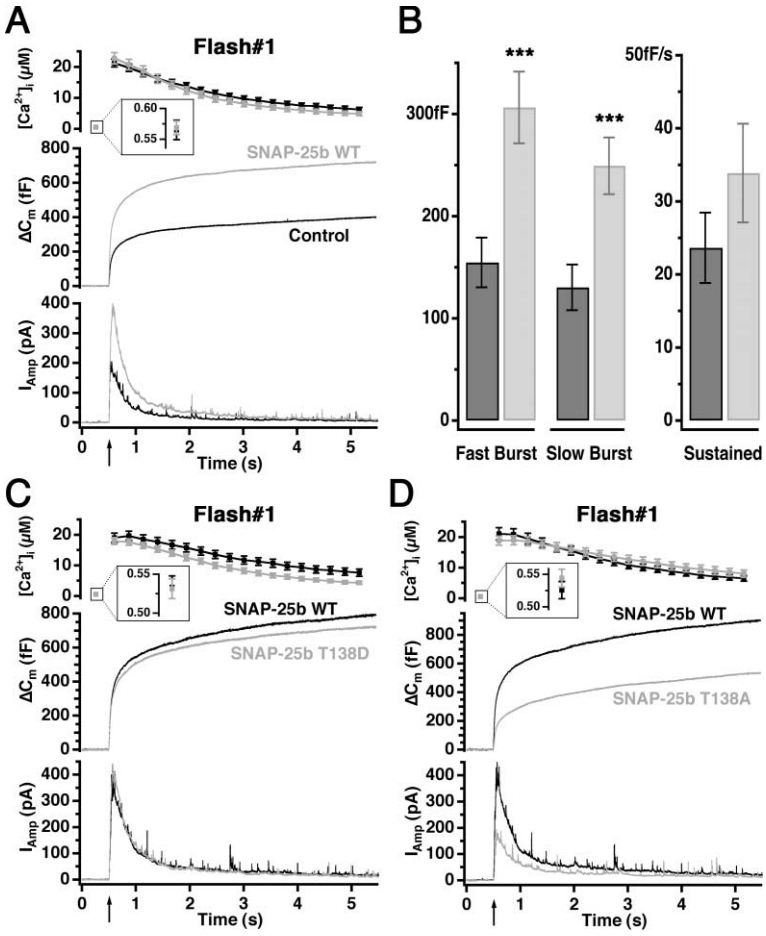

Figure 5. The T138A Mutant also Reduces the Exocytotic Burst Size when Introduced in the SNAP-25b Isoform

(A) Overexpression of wild-type SNAP-25b (gray traces; n = 32) led to an increased exocytotic response as compared to noninfected control cells (black traces; n = 34).

(B) Kinetic analysis of the experiment shown in (A). Overexpression of wild-type SNAP-25b (gray bars) increased the size of both burst components by ~2-fold, whereas the sustained component was not changed significantly (black bars: control cells; ***$p < 0.001$).

(C) Overexpression of the T138D mutant of SNAP-25b (gray traces; n = 25) resulted in a similar response to the flash as overexpression of wild-type SNAP-25b (black traces; n = 23).

(D) Overexpression of the T138A mutant of SNAP-25b (gray traces; n = 28) led to a reduction of the exocytotic burst comparing to wild-type SNAP-25b (black traces; n = 25). Note that while only the first flash-evoked responses are displayed, the size of the exocytotic burst evoked by the second flash was also reduced in the cells overexpressing T138A.

inhibitors (Figure 7B). Note that also in this case did overexpression of SNAP-25a T138A lead to a reduced exocytotic burst compared to SNAP-25a T138D overexpression (compare Figures 7A and 7B). Detailed kinetic analysis was carried out in order to identify the exocytotic component(s) affected by PKA inhibition in this case. In order to compare the two conditions, which exhibited very different burst sizes due to the effect of the mutations, we normalized pool sizes to the amplitude found in overexpressing cells without PKA inhibition (Figure 7C). This analysis revealed that inhibition of PKA selectively reduced the RRP size by ~40%–50%, whereas the size of the SRP now was unaffected by PKA inhibitors (Figure 7C). To exclude the possibility that the remaining effect on the RRP was caused by an unspecific effect of the PKA inhibitor KT5720 (Davies et al., 2000), we applied another combination of PKA inhibitors in cells overexpressing the T138D mutant (3 min intracellular perfusion with 1 μM PKI and 500 μM Rp-cAMPS). This drug combination also selectively reduced the size of the RRP by 40% (data not shown), indicating that the reduction of the RRP size is due to the inhibition of PKA. Thus, overexpression of either SNAP-25a mutant, both of which would eliminate dynamic PKA-dependent phosphorylation at position 138, occluded the effect of PKA inhibitors on the SRP, but not the RRP. These results provide evidence that phosphorylation of SNAP-25 at T138 is required for the constitutive effect of PKA on SRP size and that in addition another downstream PKA target is involved in setting the relative sizes of the RRP and the SRP (see Discussion).

Discussion

Catecholamine Release and cAMP

In this study, we have identified a constitutive stimulatory effect of PKA on secretion from chromaffin cells (Figure 1). As we outlined in the Introduction, the inconsistent results in the literature on cAMP-dependent stimulation of chromaffin cells may be explained by the fact that cAMP leads to increases in basal $[Ca^{2+}]_i$ and vice versa. Therefore, if cells are studied where the basal $[Ca^{2+}]_i$ is rather low, then cAMP might stimulate secretion. However, if cells are studied under conditions where the basal $[Ca^{2+}]_i$ is already high, exogeneous cAMP may have no further effect. We bypassed this problem by adjusting the basal $[Ca^{2+}]_i$ to the same level in each experiment before stimulating the cells. With this stimulation regime, infusion of cAMP and forskolin had no effect on secretion as monitored by capacitance or amperometric current measurements (Figure 1). However, under these conditions, PKA plays a constitutive role as illustrated by the reduction in the size of the releasable vesicle pools upon infusion of PKA inhibitors (Figure 1).

Our data are reminiscent of those of Takahashi et al.

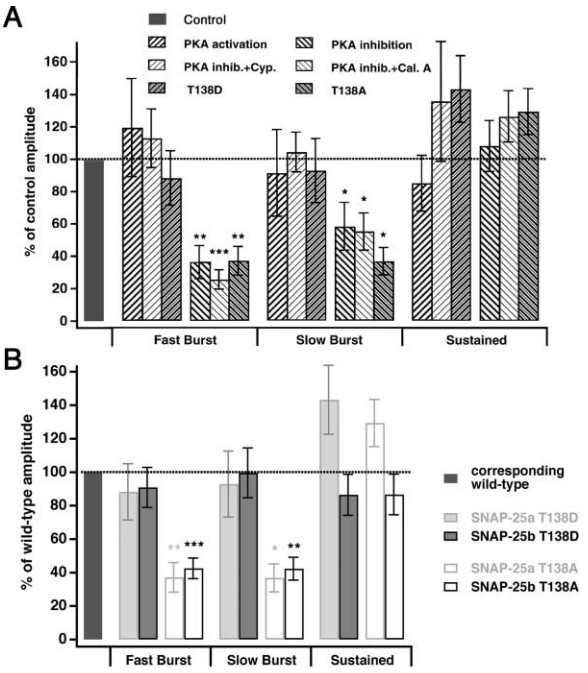

Figure 6. Kinetic Analysis of the Effect of Pharmacological Manipulations and Phosphorylation Mutants on the Exocytotic Components

(A) The bar diagram shows the normalized amplitudes of the different kinetic components obtained from fitting a triple exponential function to the capacitance responses of the first flash stimulation from individual cells. The amplitudes were normalized to the amplitude of the corresponding exocytotic component from control cells from the same cell preparations. PKA activation as well as overexpression of the SNAP-25 T138D mutant had no significant effect on any of the kinetic components. However, inhibition of PKA and overexpression of the T138A mutant significantly reduced both burst components without affecting the sustained phase. Inhibition of calcineurin with cypermethrin (Cyp) completely prevented the reduction of secretion by PKA inhibition, whereas inhibition of protein phosphatases 1 and 2A with calyculin A (Cal. A) did not. In summary, we distinguished two groups of experiments: PKA inhibition (in the presence or absence of calyculin A) and overexpression of T138A mutant reduced the fast and slow burst amplitudes by ~50%, whereas PKA activation, pretreatment of PKA-inhibited cells with cypermethrin, and overexpression of T138D mutant resulted in secretion indistinguishable from control cells. Note that the second flash-evoked responses display similar statistics.

(B) The effect of phosphorylation mutants of the two SNAP-25 splice variants compared to the corresponding wild-type splice variant (noninfected cells for SNAP-25a). T138A mutant of both splice variants induces a ~60% reduction in the size of both burst components. Despite the large variability, none of the mutants significantly changed the sustained component significantly (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

(1999), who reported that in pancreatic β cells PKA was constitutively active when the intracellular ATP concentration was high (3 mM), but not at low ATP concentrations. Notably, PKA activity was necessary for the fast part of secretion, indicating that PKA activity maintained the pool of releasable vesicles.

Antagonistic Effect of PKA and Calcineurin

Preincubation with cypermethrin, an inhibitor of the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin, blocked the effect of PKA inhibitors (Figure 2), indicating that calcineurin counteracted the process stimulated by PKA. The normal first and second flash stimulation in the simultaneous presence of calcineurin inhibitors and PKA inhibitors (Figure 2C and data not shown) show that neither PKA nor calcineurin activity is essential for vesicle pool (re)filling, but their relative activities act to modulate the SRP and RRP sizes. The simultaneous recording of capacitance and amperometric signals confirmed that the effect of calcineurin was to rescue secretion from PKA inhibition and was not caused by a change of endocytosis. Previous data implicated calcineurin in endocytosis in chromaffin cells (Artalejo et al., 1996; Engisch and Nowycky, 1998; Chan and Smith, 2001). In contrast, in several neurons and neurosecretory cells, a negative effect of calcineurin on exocytosis has been established, even though in most cases calcium influx or excitability, rather than the exocytotic machinery, were implicated (Antoni et al., 1993; Nichols et al., 1994; Sihra et al., 1995; Victor et al., 1995; Sistiaga and Sánchez-Prieto, 2000). In pancreatic α and β cells, activation of G_i-coupled receptors by somatostatin activates calcineurin and inhibits secretion (Renström et al., 1996; Gromada et al., 2001). Interestingly, in rat pancreatic α cells, calcineurin activation caused a decrease in the size of the readily releasable vesicle pool (RRP) in the presence, but not in the absence, of forskolin. The calcineurin-induced decrease in RRP size exactly cancelled the cAMP-induced increase in RRP size. This indicates that in both rat pancreatic α cells (Gromada et al., 2001) and chromaffin cells (present study) calcineurin causes depriming of vesicles, whereas PKA is responsible for the opposite effect. However, the differences between the experimental series must also be stressed: in α cells it was concluded that priming involves the movement of granules close to L-type calcium channels, so that priming is both a maturation and a localization property of the vesicles (Gromada et al., 1997). In our experiments, by using flash photolysis of caged Ca^{2+}, we studied the vesicular maturation steps in isolation.

PKA-Dependent Phosphorylation of SNAP-25

Using a phosphopeptide-specific antibody, we showed that endogeneous SNAP-25 is phosphorylated by PKA at T138 in chromaffin cells (Figure 3), extending previous findings (Risinger and Bennett, 1999; Hepp et al., 2002). Overexpression studies showed that the SNAP-25 mutant T138D, which we speculated may mimic the phosphorylated form of SNAP-25, did not modify secretion significantly (Figures 4 and 6). In contrast, the mutant T138A caused a 2-fold decrease in both exocytotic burst components (Figures 4 and 6). Furthermore, we found that overexpression of either mutant eliminated the effect of PKA inhibitors on the SRP, but not on the RRP (Figure 7). This shows that phosphorylation of SNAP-25 underlies PKAs action on the SRP. In chromaffin cells, depletion of the RRP is followed by refilling from the SRP, indicating a linear maturation scheme (Figure 7D; Voets et al., 1999). In this scheme, phosphorylation of SNAP-25 will not only regulate the size of the SRP, but also indirectly the size of the RRP. Thus, upon overexpression of SNAP-25 T138A, the size of the RRP decreased (Figure 6), due to the reduction of the SRP size.

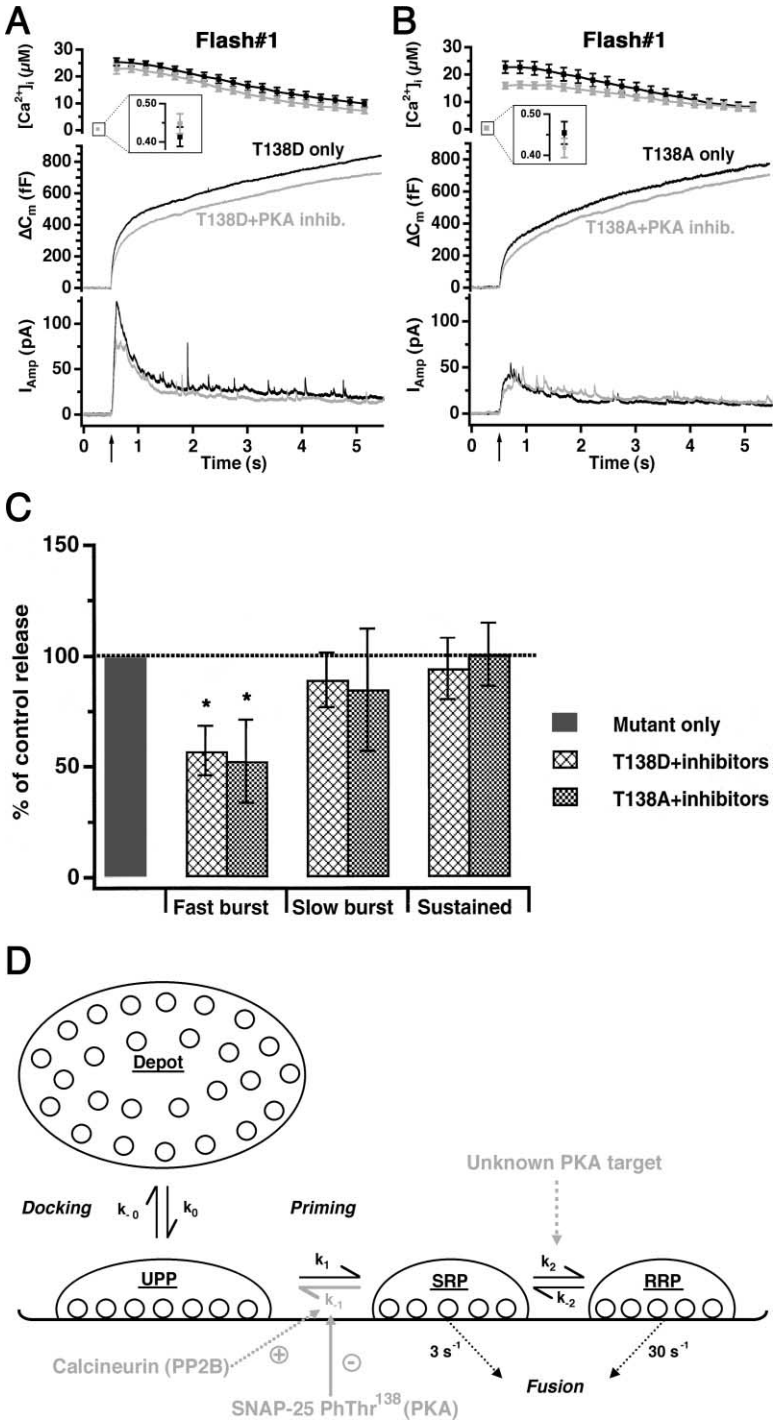

Figure 7. Overexpression of SNAP-25a Mutated at T138 Eliminates the Effect of PKA Inhibitors on the Size of the SRP

(A) Inhibition of PKA (3 min intracellular perfusion with 1 μM PKI 6–22 and 1 μM KT5720) led to only a moderate decrease of exocytotic burst if cells overexpressed the T138D mutant (gray traces; n = 19). Black traces represent mean values of T138D-expressing cells without PKA inhibition (n = 16).

(B) Overexpression of the T138A mutant resulted in a smaller exocytotic burst than the T138D mutant (compare with A), in agreement with the previous experimental series (compare with Figure 4). A moderate additional reduction was observed in cells where T138A overexpression was combined with PKA inhibitors (gray traces; n = 16) compared to untreated cells overexpressing T138A (black traces; n = 18).

(C) Pool sizes obtained by fitting the individual traces with triple exponential function were normalized to the size in overexpressing cells without PKA inhibition. Inhibition of PKA selectively reduced the size of RRP by ~40%–50% in both cases, while the SRP remained unaffected (*$p < 0.05$).

(D) A linear model of vesicle maturation, including docking, priming, and fusion according to Ashery et al. (2000). UPP, docked but not fusion competent vesicles (unprimed pool); SRP, slowly releasable pool; RRP, readily releasable pool. Primed vesicles in SRP are fusion competent, but they can mature further into the RRP and fuse with a higher rate constant. Our data show that PKA-dependent phosphorylation of SNAP-25 regulates the priming step filling the SRP, which also leads to an indirect effect on the RRP. An additional PKA target regulates the maturation step between the SRP and RRP. According to the model, PKA-dependent phosphorylation of SNAP-25 reduces the depriming rate (k_{-1}), leading to an increased size of both SRP and RRP, whereas calcineurin activity has the opposing effect. For alternative explanations, see the text.

The finding that after overexpression of T138 mutants PKA inhibitors still reduced the size of the RRP indicates the existence of an additional PKA target regulating the maturation step between the SRP and the RRP (Figure 7D).

If phosphorylation of SNAP-25 controls the step refilling the SRP, why is the sustained component of release unchanged by mutations or PKA inhibition? The sustained component is usually assumed to represent refilling of the releasable vesicle pools, followed immediately by fusion as long as the calcium concentration stays high enough after the flash (see Figure 7D). The forward priming/refilling rate, k_1, is calcium dependent (Voets, 2000), and after the flash the sustained rate approximates the maximal value of k_1 at high $[Ca^{2+}]_i$ (because the fusion rate from the SRP $>>$ k_{-1}). Obviously, this maximal rate is unchanged by mutations or PKA inhibition. Since the size of the SRP and RRP pools are determined by k_1/k_{-1}, this leaves the possibility that phosphorylation of SNAP-25 controls the depriming rate, k_{-1}, such that primed vesicles are more unstable (k_{-1} higher) in the presence of PKA inhibitors or following expression

of the T138A mutation. However, it should be stressed that so far we have not been able to measure the stability of primed vesicles directly. Another possibility is therefore that the mutations/inhibitors have changed the calcium dependence of k_1 without changing the maximal rate. Finally, it is possible that part of the sustained release component does not correspond to refilling of the SRP and RRP, but rather to slow release from yet another releasable vesicle pool (for instance, the highly calcium-sensitive pool, HCSP; Yang et al., 2002). If this is the case, manipulations that slow down refilling of the SRP could have a smaller effect on the sustained component than on SRP size.

Since calcineurin counteracts the effect of PKA in physiological experiments (Figure 2), a simple interpretation would be that calcineurin dephosphorylates the target protein phosphorylated by PKA, one of which appears to be SNAP-25. However, in experiments using our phosphopeptide-specific antibody, we could not detect more phosphorylated SNAP-25 in the presence of calcineurin inhibitors (not shown). It is therefore possible that calcineurin acts to deprime vesicles through another target protein. Another discrepancy between biochemical and physiological experiments is that in biochemical experiments, the majority of SNAP-25 was not phosphorylated in the basic state, whereas the results from physiological experiments clearly indicate that the functional pool of SNAP-25 was phosphorylated during our standard stimulation protocol. Especially the fact that overexpression of SNAP-25 mutants eliminated the effect of PKA inhibitors on the SRP size constitutes strong evidence that the functional SNAP-25 was phosphorylated. One possibility is that PKA- and calcineurin-dependent cycles of phosphorylation and dephosphorylation are local events, which only affect a small fraction of SNAP-25 molecules, possibly coordinated through the colocalization of PKA and calcineurin by binding to A-kinase anchoring protein (Coghlan et al., 1995). In addition, it should be remembered that in photolysis experiments the basal $[Ca^{2+}]_i$ was clamped to 400–600 nM, which could cause activation of PKA and also activation of calcineurin through binding of Ca^{2+}-calmodulin, whereas during biochemical experiments the resting $[Ca^{2+}]_i$ would be in the 50–100 nM range (Nagy et al., 2002). Thus, a perfect correlation between biochemical and physiological experiments cannot be expected.

The other PKA target, which acts at the maturation step between the SRP and the RRP (Figure 7D), is so far unidentified. It is known that this step requires the presence of synaptotagmin 1, since in its absence the RRP, but not the SRP, is missing (Voets et al., 2001). Since synaptotagmin 1 itself is not PKA phosphorylated (Hilfiker et al., 1999), candidates for this PKA target are proteins interacting with synaptotagmin 1, for instance cysteine string protein (Evans and Morgan, 2002), which is a major target for PKA phosphorylation (Evans et al., 2001), or snapin (Chheda et al., 2001). Indeed, it was shown that a phosphorylation mutant of snapin increases the size of the exocytotic burst in chromaffin cells (Chheda et al., 2001).

Physiological Implications

We previously showed that block of endogeneous PKC activity in chromaffin cells has no effect on the first flash stimulation, but causes a decreased response to the second stimulation (Nagy et al., 2002). This effect was mirrored by overexpression of SNAP-25 mutated in the PKC phosphorylation site S187, located in the C-terminal end of the molecule. In the present study, all manipulations, except for calyculin A treatment (Figure 2B), had similar effects on the first and second stimulations. Thus, under our recording conditions, PKA (and phosphorylation of SNAP-25 at T138) controls the size of the releasable vesicle pools, whereas PKC activity (and phosphorylation of SNAP-25 at S187) is involved in regulating refilling after the pools have been emptied. The effect of calyculin A on the second, but not the first, stimulation is consistent with a function of protein phosphatase 1 or 2A to counteract the effect of PKC. Another possibility is, however, that because of the short preincubation time (5 min), the calyculin effect developed during the experiment.

Overexpression of the brain-specific splice variant SNAP-25b in bovine chromaffin cells increased the size of the SRP and RRP by a factor of 2 (Figure 5A), a result similar to that in mouse chromaffin cells (Sørensen et al., 2003). The effect of T138 mutation to alanine was a 2-fold decrease in SRP and RRP size, whether the mutation was introduced in SNAP-25a (Figure 4) or SNAP-25b (Figure 5D, see also Figure 6B). These data show that the effects of T138 mutation (and, by inference, T138 phosphorylation) and SNAP-25 isoform are independent of each other. In the assembled SNARE complex, the T138 site is located at the far end away from the membrane anchors, whereas the amino acid differences between SNAP-25 a and b isoforms are located in the SNARE complex half closest to the membrane anchors, and in the linker between the two SNARE motifs in SNAP-25. Thus, the two manipulations have similar effects though they are located in separate halves of the SNARE complex.

We can conclude that the cell has two different ways of regulating the size of the releasable vesicle pools, without affecting the rate of sustained secretion: a pretranslational (alternative splicing) and a posttranslational (phosphorylation), both of which act on SNAP-25. This provides the cell with maximal flexibility to modulate exocytotic strength in response to acute as well as longer-lasting or chronic (e.g., developmental) changes in physiological condition.

Experimental Procedures

Generation and Purification of Antibodies

Antibodies directed against phosphorylated SNAP-25 were raised to a peptide with the sequence H_2N-CFIRRV(pT)NDARE-$CONH_2$, equivalent to the SNAP-25 residues 133–143, including phosphothreonine at position 138 (Eurogentech, Seraing, Belgium). For affinity purification, 10 mg of phosphopeptide antigen were conjugated with thiopropyl sepharose 6B (0.5 g; Amersham Pharmacia Biotech, Piscataway, NJ). The phosphopeptide-conjugated resin was incubated with 5 ml of antiserum and then washed extensively first with 0.1 M Tris (pH 8.0), followed by 0.5 M NaCl/0.1 M Tris (pH 8.0) and 0.01 M Tris (pH 8.0). Bound antibodies were eluted with 0.2 M glycine (pH 2.7)/0.1 M NaCl, followed by 0.2 M glycine (pH 2.3)/0.1 M NaCl, and immediately neutralized with Tris-HCl. Purified antibodies were concentrated with Centricon 30.

Generation of Mutant SNAP-25 Constructs and Expression in HEK293 Cells

SNAP-25 T138A mutant construct was generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

The expression constructs were verified by sequencing. HEK293 cells were maintained in DMEM (Invitrogen, San Diego, CA) containing 5% (v/v) of fetal calf serum (PAA Laboratories, Pasching, Austria), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Cells (40%–80% confluent) were transfected with 10 μg of expression vectors encoding wild-type SNAP-25 or SNAP-25 T138A using the SuperFect Transfection Reagent (QIAGEN, Hilden, Germany). One day after transfection, cells were incubated with PKA activators or PKA inhibitor for 20 min. Cells were then washed once with ice-cold PBS and harvested in PBS. After trituration, the protein concentration of the extracts was measured with a Bradford assay and SDS-PAGE was performed with 40 μg of each extract on 18% acrylamide gels according to standard methods (Laemmli, 1970). Separated proteins were blotted to nitrocellulose and analyzed by immunoblotting with the phosphospecific anti-SNAP-25 antibody (1:20) as well as a monoclonal anti-SNAP-25 antibody (clone 71.1; Synaptic Systems, Göttingen, Germany; 1:1,000,000) and ECL (Amersham Pharmacia Biotech).

Phosphorylation Experiments and Protein Analysis

For the analysis of PKA-dependent phosphorylation of SNAP-25 in cultured chromaffin cells, 0.5–1 million bovine chromaffin cells were plated onto poly-D-lysin-coated 5 cm dishes, and experiments were carried out 2 days after preparation. Cells were incubated in external solution identical to that used for electrophysiological experiments in the presence of kinase activators and inhibitors for 20 min at 37°C and with 8% CO_2. After treatment, cells were harvested in extraction buffer (1% sodium cholate, 2 mM EGTA, 25 mM HEPES-KOH [pH 7.4], 100 mM NaCl, 1 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride). Protein concentrations were measured with a Bradford assay, and SDS-PAGE was performed with 50 μg of each extract on 18% acrylamide gels according to standard methods. Separated proteins were blotted to nitrocellulose and analyzed by immunoblotting with the phosphospecific anti-SNAP-25 antibody (1:20) as well as a monoclonal anti-SNAP-25 antibody (clone 71.1; Synaptic Systems, Göttingen, Germany; 1:1,000,000) and ECL (Amersham Pharmacia Biotech).

Chromaffin Cell Preparation and Electrophysiological Recordings

Bovine chromaffin cell preparation, whole-cell patch-clamp capacitance, amperometry, and calcium measurements were performed as described previously (Nagy et al., 2002). Nitrophenyl-EGTA was supplied by G. Ellis-Davies (MCP Hahnemann University, Philadelphia, PA). 8-Br-cAMP (Calbiochem, Bad Soden, Germany), cAMP (Sigma-Aldrich, Steinheim, Germany), Rp-cAMPS (Calbiochem), and PKI 6–22 (Calbiochem) were diluted in water as stock solutions at 250 mM, 1 mM, 5 mM, and 200 μM, respectively. Stock solutions of forskolin (100 mM, Calbiochem), 3-isobutyl-1-methylxanthine (IBMX; 200 mM, Calbiochem), and KT5720 (10 mM, Calbiochem) were prepared in DMSO. Calyculin A (100 μM, Calbiochem) and cypermethrin (10 mM, Calbiochem) were diluted in 70% ethanol.

For kinetic analysis, individual capacitance traces were fitted with a triple exponential function:

$$f(t) = A_0 + \sum_{i-1}^{3} A_i \cdot (1 - \exp(-(t - t_0)/\tau_i)) \qquad \text{for } t > t_0,$$

where A_0 is the capacitance of the cell before the flash and t_0 is the time of the flash. The amplitudes (A_i) and time constants (τ_i) of the two faster exponentials define the size and release kinetics of the SRP and the RRP, respectively, whereas the slowest exponential was included in order to correct for the sustained component of release. Since the time constant of the sustained component is too slow to be measured accurately in this way, this third exponential was not used directly. Instead, we subtracted the amplitudes of the fast and slow burst from the total amount of secretion during 5 s and calculated the linear rate of sustained release.

Statistical testing of the amplitudes and time constants of the kinetic components was performed by Mann-Whitney U test. Numbers given in the text represent mean ± SEM. Each experimental condition was compared to control cells obtained in parallel from the same cell preparations.

Plasmid and Virus Generation for Overexpression in Chromaffin Cells

For construction of GFP-SNAP-25a fusion construct, an oligonucleotide cassette was introduced into the XmaI site of the viral vector pSFV1 (Invitrogen) to generate singular ClaI and BssHII restriction sites. The gene coding for GFP was amplified by PCR to generate a 5′ BglII and 3′ BamHI site and inserted into the BamHI site of of the modified pSFV1. The coding sequence for rat SNAP-25a was cloned into the BamHI-ClaI-cleaved pSFV1-GFP. Murine SNAP-25b was cloned into a modified pSFV1 plasmid, where an internal ribosome entry site was inserted followed by the gene for enhanced GFP, as described (Sørensen et al., 2002). SNAP-25 mutants were generated by site-directed mutagenesis. The sequence of all constructs was verified by DNA sequencing. Virus production and infection was performed as described previously (Ashery et al., 1999).

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