Soluble *N*-ethylmaleimide-sensitive-factor attachment protein and *N*-ethylmaleimide-insensitive factors are required for Ca^{2+} -stimulated exocytosis of insulin

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Ca^{2+} stimulates exocytosis in permeabilized insulin-secreting cells. To investigate the putative cytosolic components involved in the Ca^{2+} response, HIT-T15 cells (a pancreatic B-cell line) were permeabilized with streptolysin-O, a procedure that allows rapid exchange of soluble components including macromolecules. We found that in this cell preparation the secretory response to Ca^{2+} but not to guanosine 5'-[γ -thio]triphosphate was lost as a function of time and could be restored by rat brain cytosol in a concentration-dependent manner. Reconstitutive activity of rat brain cytosol was found in a high-molecular-mass heat-labile partially *N*-ethylmaleimide (NEM)-sensitive fraction. The NEM-

sensitive factor (NSF) and the soluble NSF attachment protein (α -SNAP) were found to be expressed in HIT-T15 cells and largely lost (about 30 % remaining) from porated cells. Recombinant α -SNAP partially reconstituted the Ca^{2+} response when added to the permeabilized cells. Moreover, α -SNAP restored the effect of NEM-treated cytosol to the level observed for untreated cytosol. In contrast, NSF was ineffective when preincubated alone or with NEM-treated cytosol. Our results indicate that both α -SNAP and NEM-insensitive cytosolic factors are involved in Ca^{2+} -mediated exocytosis from endocrine HIT-T15 cells.

INTRODUCTION

The regulation of insulin secretion from the pancreatic B-cells is the single most important factor controlling blood glucose homeostasis. Glucose and other secretagogues induce elevation of intracellular free Ca^{2+} concentrations, which represents one of the main triggering events of insulin secretion [1,2]. Exocytosis constitutes the last stage of the intracellular vesicular vectorial transport, when a granule fuses with the plasma membrane to release its contents into the extracellular space. *In vitro* reconstitution of intracellular vesicle trafficking [3] and genetic studies in yeast [4,5] revealed that the mechanisms underlying secretion are conserved from yeast to mammals [6] and led to the identification of the *N*-ethylmaleimide (NEM)-sensitive factor (NSF) [7] and soluble NSF attachment proteins (SNAPs) [8]. Both proteins were shown to be essential for several intracellular transport steps [9]. Subsequent to the purification of the receptors for the SNAPs on both vesicles and target membranes (respectively named v- and t-SNARE), the following model known as the SNARE hypothesis [10] was proposed: in neurons and neuroendocrine cells the docking of a vesicle with a membrane would result from the specific interaction between a vesicle receptor (v-SNARE) and its cognate receptor (t-SNARE) on the target membrane. The further association of the cytosolic factors α -SNAP and NSF to form a 20 S protein complex would lead to the fusion event, possibly through the ATPase activity of NSF. Several components of the neuronal fusion machinery have been identified in pancreatic islets [11]. A functional role for at least two of these proteins, the v-SNARE VAMP-2 [12] and the t-SNARE SNAP-25 [13], has now been shown in insulin secretion. However, the molecular mechanisms that trigger exocytosis from endocrine B-cells remain unclear.

Characterization of the factors controlling exocytosis has been facilitated by the creation of pores in the cytoplasmic membrane of cells allowing access to the intracellular environment. Detergents such as digitonin [14] and toxins such as streptolysin-O (SL-O) [15,16] produce large-size pores (diameter > 13 nm). Such permeabilized cells retain a good initial response to Ca^{2+} but display a decline in the secretory response as the result of the progressive loss of the cytosolic components [14]. Several soluble proteins were demonstrated to be essential for exocytosis through their ability to reconstitute part of the Ca^{2+} response when added back to permeabilized cells [17–20]. Recently, such a run-down/reconstitution assay was used to demonstrate a role for the general fusion factor α -SNAP in exocytosis from chromaffin cells [21].

In the present study, SL-O-permeabilized insulin-secreting cells were employed to characterize the soluble mediators of the Ca^{2+} response. Permeabilized HIT-T15 cells exhibited a run-down for Ca^{2+} but not for guanosine 5'-[γ -thio]triphosphate GTP[S]-mediated stimulation, in agreement with recent findings suggesting that insulin-secreting cells possess two distinct pathways for the control of exocytosis [12]. The decreased insulin response of the permeabilized cells could be completely reconstituted with rat brain cytosol providing evidence that the run-down was not due to a failure of the intracellular machinery but to the leakage of cytosolic components. Therefore SL-O-permeabilized HIT-T15 cells proved to be a system suitable for the study of the soluble components involved in Ca^{2+} -stimulated exocytosis. Characterization of the rat brain cytosol and use of recombinant proteins in run-down/reconstitution assays suggested that α -SNAP and, in addition, NEM-insensitive factors are required for Ca^{2+} -stimulated exocytosis from endocrine HIT-T15 cells.

Abbreviations used: α -SNAP, soluble NSF attachment protein; GTP[S], guanosine 5'-[γ -thio]triphosphate; NEM, *N*-ethylmaleimide; NSF, *N*-ethylmaleimide-sensitive factor; SL-O, streptolysin-O toxin; SNARE, SNAP Receptors.

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MATERIALS AND METHODS

Cell culture, cell permeabilization and insulin secretion

Insulin-secreting HIT-T15 cells were cultured in RPMI 1640 medium as described previously [16] for 2–3 days in 96-well plates (10^5 cells/200 μ l per well). On the day of the experiment, the cells were washed once with Krebs–Ringer buffer (136 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 5 mM NaHCO_3 and 25 mM Hepes, pH 7.4) and then once with potassium glutamate buffer (140 mM potassium glutamate, 5 mM NaCl, 7 mM MgSO_4 , 0.4 mM EGTA, 20 mM Hepes, pH 7.0). Thereafter cells were permeabilized with SL-O in potassium glutamate buffer for 7 min, resulting in at least 95% of cells being permeable to Trypan Blue. Permeabilization and the different incubation steps were performed at 37 °C. SL-O was produced and purified as described previously [22]. After removal of the permeabilizing solution, cells were preincubated for various times in potassium glutamate/EGTA/ CaCl_2 buffer resulting in a final concentration of 0.1 μ M free Ca^{2+} and containing 2.5 mM ATP in the presence or absence of the test agents. Secretion of insulin was subsequently measured after incubating the cells with potassium glutamate/EGTA/ATP/ CaCl_2 buffer containing 0.1 or 10 μ M free Ca^{2+} for 7 min at 37 °C without agents to be tested. The media were removed, centrifuged at 4 °C and the supernatants kept at –20 °C until insulin assay. Attached cells were extracted with acid/ethanol for the determination of insulin content. Insulin was measured by radioimmunoassay using human insulin as standard. Concentrations of free Ca^{2+} were determined with a Ca^{2+} -sensitive electrode.

Preparation of cytosol

Rat brains were excised and washed several times with Krebs–Ringer buffer and glutamate buffer containing 1 mM dithiothreitol, 4 μ g/ml leupeptin and 10 μ g/ml aprotinin before homogenization; the homogenate was then centrifuged for 10 min at 3000 *g* (5000 rev./min), at 4 °C and the postnuclear supernatant was ultracentrifuged at 100000 *g* (45000 rev./min) at 4 °C for 90 min; the supernatant was taken as the cytosol. Cytosolic fractions were obtained by centrifuging cytosol through Centricon microfilter concentrators (Amicon, Wallisellen, Switzerland) for 120 min (4000 *g*; 4 °C). Protein content in the cytosol was determined by Coomassie Blue binding using BSA as standard.

Recombinant α -SNAP and NSF and Western-blot analysis

Plasmids encoding His₆-tagged NSF and α -SNAP were generously donated by Dr. J. E. Rothman (Memorial Sloan-Kettering Cancer Center, New York, NY, U.S.A.). Anti- α -SNAP serum raised against recombinant His₆- α -SNAP was kindly provided by Dr. T. Levine (ICRF Laboratories, London, U.K.). His₆-tagged α -SNAP and NSF were purified using standard procedures [23].

Samples for Western-blot analysis were separated by SDS/PAGE as described [24], transferred to poly(vinylidene difluoride) and incubated with a rabbit polyclonal antibody against recombinant α -SNAP (1:3000) or recombinant NSF (1:6000). Visualization of immunoreactivity was performed using enhanced chemiluminescence reagents (Amersham International, Amersham, Bucks., U.K.). α -SNAP content of HIT-T15 cells and rat brain cytosol was quantified by comparison with different concentrations of recombinant α -SNAP and scanning the autoradiographs with a Computing Densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The concentration curve of recombinant α -SNAP was linear in the tested range from 0.25 to 2 ng.

Statistics

Results are presented as means $\pm$ S.E.M. from at least three separate experiments. Statistical analysis was performed by Student's two tailed *t* test for unpaired data (*P*).

RESULTS

Run-down of the Ca^{2+} response in permeabilized HIT-T15 cells

Validation of permeabilized cells as a tool for run-down/reconstitution assays depended on the integrity of the exocytotic machinery and the specificity of the Ca^{2+} response. Therefore SL-O-permeabilized HIT-T15 cells were stimulated with 10 μ M Ca^{2+} or 100 μ M GTP[S] after different preincubation times (Figure 1, top). Insulin release in response to 100 μ M GTP[S] exhibited a maximal 2–3-fold increase over basal release and displayed no run-down. When Ca^{2+} was added during SL-O permeabilization (time point 0 min), a 15–20-fold increase in the secretory response was observed. This stimulation was lost over

Figure 1 Time-dependent run-down of the effect of Ca^{2+} on insulin secretion

HIT-T15 cells were permeabilized for 7 min with SL-O. Stimulation with 0.1 μ M Ca^{2+} ($\circ$), 10 μ M Ca^{2+} ($\square$) or 100 μ M GTP[S] ($\triangle$) was performed either during permeabilization ($t = 0$ min), directly after SL-O treatment ($t = 7$ min) or after 20 min preincubation ($t = 27$ min). The cellular insulin content was 53.20 ± 7.36 ng ($n = 14$). Top, values ($n = 3-12$) are given as percentage of the insulin content per well. * $2P < 0.05$ as compared with the immediate response (0 min) or the stimulation after 7 min permeabilization (7 min). Bottom, experiments were performed as above, except that cytosol (0.75 mg/ml), when present ($\blacksquare$), was added during permeabilization (7 min) or during the 20 min preincubation but not during stimulation. Values ($n = 6-17$) are given as percentage of the insulin content per well. * $2P < 0.05$ compared with the absence of cytosol ($\square$).

Figure 2 Dose-dependent effect of cytosol

HIT-T15 cells were permeabilized for 7 min with SL-O and either directly stimulated with $10 \mu\text{M}$ Ca^{2+} or preincubated for 20 min in the presence of different concentrations of cytosol from the indicated sources followed by stimulation in the absence of cytosol. To normalize the values, 100% reconstitution was defined as the difference between Ca^{2+} -stimulated insulin release directly after SL-O permeabilization (see Figure 1, time point 7 min) and after 20 min preincubation (see Figure 1, time point 27 min) $^*P < 0.05$ compared with the absence of cytosol.

Table 1 Reconstitution activity of cytosolic factors

HIT-T15 cells were permeabilized with SL-O for 7 min and directly stimulated with $10 \mu\text{M}$ Ca^{2+} (stimulation after 7 min) or preincubated for 20 min in the absence or presence of cytosol (0.75 mg/ml), microfiltrated cytosol ($> 100 \text{ kDa}$, $< 100 \text{ kDa}$), heated cytosol (10 min at 60°C) or BSA; stimulation with $10 \mu\text{M}$ Ca^{2+} was performed in the absence of cytosol or BSA. As detailed in Figure 2, values ($n = 3-36$) are given as percentage of reconstitution of the response after SL-O permeabilization. * Compared with the absence of cytosol; † compared with non-treated cytosol.

Time before stimulation (min)	Agents added during preincubation	Reconstitution of the initial response (%)		
		Mean $\pm$ S.E.M.	<i>n</i>	<i>2P</i>
7	—	100 ± 3	36	$< 0.01^*$
27	—	0 ± 2	29	
27	Non-treated cytosol	127 ± 12	12	$< 0.01^*$
27	Filtrated cytosol ($> 100 \text{ kDa}$)	92 ± 12	4	$< 0.01^*$
27	Filtrated cytosol ($< 100 \text{ kDa}$)	29 ± 7	5	$< 0.01^\dagger$
27	Heated cytosol	6 ± 2	6	$< 0.01^\dagger$
27	BSA	0 ± 1	3	

time, and the run-down started immediately with a rapid 50% decrease of the response during the first 7 min of permeabilization. A slower decrease was observed during the following 20 min (time point 27 min). In initial attempts, rat brain cytosol failed to restore the Ca^{2+} effect on secretion. This was probably due to the proteolysis of secreted insulin, as in the absence of cells a reduced recovery of even exogenously added purified insulin was evident in the presence of brain cytosol (results not shown). Therefore cytosol was added only during the preincubation and not during the final stimulation period. Under these conditions brain cytosol considerably retarded the run-down (Figure 1, bottom). Rat brain cytosol was initially chosen as being a source probably enriched in exocytosis-mediating proteins. This reconstitutive activity of brain cytosol was not mediated by extracellular receptors since cytosol had no effect on intact cells (results not shown). This indicated that the progressive decrease in the response to Ca^{2+} was due to the loss of cytosolic proteins and not to the failure of the system or to a non-specific

Figure 3 Demonstration of the presence of α -SNAP and NSF in HIT-T15-cell cytosol and their partial loss from permeabilized cells

(A) Cytosols from rat liver (L, $30 \mu\text{g}$), HIT-T15 cells (H, $30 \mu\text{g}$) or brain (B, $30 \mu\text{g}$) were separated by SDS/PAGE, transferred to poly(vinylidene difluoride) and incubated with a polyclonal antibody against recombinant α -SNAP (1:3000) or recombinant NSF (1:6000). (B) Intact (lane 1) HIT-T15 cells were solubilized in sample buffer, or permeabilized for 7 (lane 2) or 27 min (lane 3) in the presence or absence of ATP before solubilization and treatment as in (A).

extracellular effect. Lack of run-down in response to GTP[S] suggested that the action of Ca^{2+} was selectively dependent on cytosolic factors.

Reconstitutive activity of rat brain cytosol

In the following experiments we tried to characterize the reconstitutive activity present in rat brain cytosol. Rat brain cytosol, when present, was added during the 20 min preincubation period, i.e. after exposure to SL-O, but not during stimulation. In this setting, a dose-dependent reconstitutive effect with a half-maximal effect at 0.4 mg/ml was observed (Figure 2). In parallel experiments, cytosol prepared from HIT-T15 cells was less effective, even at higher concentrations. Moreover, cytosol from liver, an organ devoid of regulated secretion, was unable to reconstitute the Ca^{2+} response, even when higher doses were used (results not shown). To estimate the molecular size of the soluble factors with reconstitutive activity, rat brain cytosol was centrifuged in microfilters with different cut-off size. Fractions above 100 kDa completely reconstituted the Ca^{2+} response (Table 1) as did untreated cytosol. In contrast, the fraction below 100 kDa exhibited only minor effects. Consistent with the idea that specific proteins are involved in Ca^{2+} -mediated exocytosis, heating (10 min, 60°C) of complete or filtered cytosol abolished the reconstitution effect. Accordingly, BSA used as control was unable to replace cytosol.

NEM-sensitive and -insensitive soluble factors implicated in the Ca^{2+} response

Using *in vitro* reconstitution assays, the NEM-sensitive factor NSF and α -SNAP have been implicated in multiple fusion events in the cell and have been shown to form a fusion complex with the membrane proteins VAMP, syntaxin and SNAP-25 [10,25]. To test whether these cytosolic proteins are involved in exocytosis from insulin-secreting HIT-T15 cells, a series of experiments was conducted. Thus, in a first approach, we performed a Western-blot analysis with polyclonal antibodies raised against NSF and α -SNAP (Figure 3A) to detect the presence of these proteins in the different cytosols used in run-down/reconstitution assays. NSF and α -SNAP were found in brain and HIT-T15 cell cytosols. α -SNAP was detected as a single band in brain cytosol, whereas

Table 2 Effect of α -SNAP and NEM on Ca^{2+} -stimulated exocytosis

HIT-T15 cells were permeabilized with SL-O for 7 min and directly stimulated with $10 \mu\text{M}$ Ca^{2+} (stimulation after 7 min) or preincubated for 20 min in the absence or presence of rat brain cytosol (0.75 mg/ml), recombinant α -SNAP or recombinant NSF before stimulation with $10 \mu\text{M}$ Ca^{2+} in the absence of indicated components. NEM-treated cytosol was incubated for 15 min at 4°C with 1 mM NEM and then for a further 5 min with 2 mM dithiothreitol before utilization. As detailed in Figure 2, values ($n = 6$ –36) are given as percentage of reconstitution of the response after SL-O permeabilization. * Compared with the absence of cytosol; † compared with non-treated cytosol; ‡ compared with NEM-treated cytosol or recombinant proteins alone.

Time before stimulation (min)	Agents added during preincubation	Reconstitution of the initial response (%)		
		Mean $\pm$ S.E.M.	<i>n</i>	<i>P</i>
7	—	100 ± 3	36	$< 0.01^*$
27	—	0 ± 2	29	
27	Non-treated cytosol	127 ± 12	12	$< 0.01^*$
27	NEM-treated cytosol	55 ± 16	4	$< 0.01^* \dagger$
27	α -SNAP (50 nM)	26 ± 7	22	$< 0.05^*$
27	NSF (500 nM)	6 ± 3	6	
27	α -SNAP (50 nM) + NSF (500 nM)	36 ± 16	12	$< 0.05^*$
27	NEM-treated cytosol + α -SNAP (50 nM)	109 ± 21	12	$< 0.01 \ddagger$
27	NEM-treated cytosol + NSF (500 nM)	70 ± 25	3	
27	NEM-treated cytosol + α -SNAP (50 nM) + NSF (500 nM)	120 ± 22	6	$< 0.01 \ddagger$

a double band was observed in HIT-T15 cells and cytosol. α -SNAP was less abundant in brain cytosol, representing $60.8 \pm 28.7\%$ ($n = 3$) of α -SNAP content of HIT-T15 cell cytosol. In contrast, NSF was present in smaller amounts in HIT-T15 cell cytosol and, from scans of the autoradiographs, estimated to represent $49.7 \pm 18.5\%$ ($n = 3$) of brain cytosol NSF. Only extremely low levels of α -SNAP and NSF were present in liver cytosol. NSF and α -SNAP contents were then estimated in intact and SL-O-permeabilized cells by Western-blot analysis and scanning of the autoradiographs (Figure 3B). The results showed a decrease in cellular level for both proteins over time. In three different experiments the loss of NSF amounted to $25.7 \pm 14.7\%$ compared with intact cells after permeabilization ($t = 7$ min) and $67.7 \pm 11.3\%$ after a further 20 min ($t = 27$ min) in the absence of ATP. In contrast, $66.6 \pm 19.2\%$ ($n = 2$) of α -SNAP content was already lost during the 7 min permeabilization period and $73.4 \pm 20.0\%$ ($n = 2$) after 27 min in the absence of ATP. The presence of ATP did not significantly change the loss of either α -SNAP or NSF (Figure 3B).

In two experiments, α -SNAP content of intact HIT-T15 cells and brain cytosol was quantified by comparison with different concentrations of recombinant α -SNAP and scanning of the autoradiographs. HIT-T15 cells contained $0.4 \pm 0.1 \text{ ng}/10^5$ cells α -SNAP. In brain cytosol $0.44 \pm 0.01 \text{ ng}/20 \mu\text{g}$ of protein was measured. Therefore the addition of $25 \mu\text{g}$ of brain cytosol/ 10^5 HIT-T15 permeabilized cells required to reconstitute the Ca^{2+} response in run-down/reconstitution experiments (see Figure 2, 100% reconstitution with 0.5 mg/ml cytosol) approximately equalled the amount of the endogenous cellular α -SNAP. We also compared the relative amount of NSF present in brain cytosol and HIT-T15 cells. The result indicates that about a tenfold excess of exogenous NSF over NSF present in HIT-T15 cells was added during the run-down/reconstitution experiments,

when $25 \mu\text{g}$ of brain cytosol was used (see Figure 2, 100% reconstitution).

To assess the role of NSF, we took advantage of the NEM-sensitive property of this protein to evaluate its contribution in the reconstitutive activity of rat brain cytosol. Brain cytosol was treated with 1 mM NEM immediately before use in the run-down/reconstitution assays. Under this condition, NEM treatment of cytosol reduced the reconstitutive activity by 50% compared with native cytosol (Table 2). The NEM-sensitive protein NSF had no effect, whether added alone or together with α -SNAP to the permeabilized cells during the preincubation period only. In contrast with the effect of NSF, the soluble factor α -SNAP alone was capable of inducing a partial reconstitution of the Ca^{2+} response that was maximal at 50 nM. This partial reconstitution was not significantly altered by increasing the concentration of α -SNAP to $1 \mu\text{M}$ or by the addition of recombinant α -SNAP alone or with NSF during the permeabilization, preincubation and stimulation periods (results not shown). We then tested whether the recombinant proteins NSF and/or α -SNAP could recover the cytosolic activity lost on NEM pretreatment. Again NSF was unable to restore the activity of NEM-treated cytosol. Interestingly, α -SNAP (50 nM) was able to restore completely the cytosolic activity lost after NEM treatment. In this case, the effect of α -SNAP was slightly more than additive to the activity of NEM-treated cytosol. No reconstitutive effect was observed when heated cytosol or BSA instead of NEM-treated cytosol was preincubated with recombinant α -SNAP (results not shown).

DISCUSSION

Permeabilized cells are known to lose cytosolic components and their secretory response [14]. We took advantage of this phenomenon to characterize soluble proteins required for exocytosis. Although insulin-secreting HIT-T15 cells exhibit a run-down of the secretory response to Ca^{2+} over time, as reported previously [16], stimulation of exocytosis by GTP[S] was not altered by this procedure. This suggests first that the observed loss of Ca^{2+} -stimulated exocytosis represents a specific event and does not imply a general degradation of the exocytotic machinery. Secondly, it underlines the use of different mechanisms in Ca^{2+} - and GTP[S]-stimulated exocytosis. Indeed, whereas in HIT-T15 cells the former is blocked by the neurotoxins botulinum B and tetanus, which cleave the vesicle proteins VAMP-2 and cellubrevin, the latter is insensitive to the toxins [12]. Furthermore this indicates that any reconstitution of the loss of the secretory response will only interfere with specific components of the exocytotic process, i.e. the part regulated by Ca^{2+} .

Our results demonstrate that the run-down can be reconstituted by a high-molecular-mass cytosolic fraction that is heat-labile and partially sensitive to NEM. This is in contrast with *in vitro* assays of vesicular transport which are highly sensitive to NEM. Note that in our system, only the cytosol, and not the cells, was treated with NEM, as this alkylating agent modifies a number of cellular proteins, including F-actin, and produces variable responses depending on the concentration employed [26,27]. We were unable to demonstrate a requirement for NSF under these conditions. In contrast, the soluble protein α -SNAP was capable of partially reconstituting on its own. This effect completely accounted for the NEM-sensitive part of the cytosolic activity, consistent with previous observations that some of the actions of α -SNAP are particularly sensitive to NEM [28]. Similar reconstitutive effects of α -SNAP but not of NSF have been reported for chromaffin cells [21]. What might be the reasons for the only partial effect of α -SNAP and the inability of NSF to reconstitute

the Ca^{2+} -stimulated exocytosis? Our data indicate that both proteins can be dialysed in our system and a functional activity of α -SNAP is expected since this factor has been described to be rate-limiting in exocytosis from squid giant axon [29]. One explanation for the incomplete reconstitution by α -SNAP in our system is given by the faster kinetics of neurosecretion [30] when compared with endocrine exocytosis [31] which implies a more rapid turnover of the fusion proteins. Yet, in view of the incomplete losses of these cytosolic proteins from permeabilized cells, we favour the hypothesis that the remaining cellular α -SNAP and NSF are already membrane-bound and, at least for NSF, sufficient for exocytosis to occur. Indeed, the temporal sequence of the fusion event *in vivo* has not been precisely characterized and some data suggest that α -SNAP/NSF intervene before the Ca^{2+} trigger for exocytosis [32,33].

Interestingly, about 50% of the reconstitutive activity of cytosol is NEM-insensitive. Clearly, factors other than α -SNAP and NSF are required for Ca^{2+} -stimulated exocytosis of insulin. This is further underlined by the smaller reconstitutive activity of cytosol from HIT-T15 cells than to that of brain cytosol despite the presence of higher levels of α -SNAP in HIT-T15-cell cytosol. Furthermore, the difference in the content of NSF cannot account for the low efficacy of cytosol from HIT-T15 cells, since recombinant NSF itself did not exhibit any reconstitutive activity. Indeed several other cytosolic proteins have been demonstrated to be implicated in Ca^{2+} -mediated exocytosis in neuroendocrine cells, such as the Ca^{2+} /phospholipid-binding protein annexin II [17], p145 and calmodulin [34]. In fact, low levels of p145 are present in HIT-T15 cells [20], and calmodulin may play a role in stimulated exocytosis from intact cells according to pharmacological characterizations [1]. In addition, protein kinases A and C participate in the regulation of Ca^{2+} -dependent exocytosis in chromaffin cells [35]. However, a contribution of these proteins to exocytosis from SL-O-permeabilized HIT-T15 cells has been previously excluded [36].

Genetic analysis of different systems demonstrated the prime importance of the Ca^{2+} stimulation of the vesicle membrane protein, synaptotagmin, in neuroexocytosis [37]. We currently do not know whether synaptotagmin plays the same crucial role in the exocytosis of insulin, although some of its isoforms are present in insulin-secreting B-cells [11,38]. However, our data suggest that synaptotagmin will not be the only factor required but that the cytosolic components are also absolutely necessary for the effective expression of Ca^{2+} -stimulated exocytosis. Probably they do not exert their function by interacting with synaptotagmin, as the latter can induce Ca^{2+} -dependent vesicle aggregation in the absence of other proteins [39].

In conclusion, we have demonstrated that the loss of stimulus-secretion coupling in permeabilized endocrine cells can be used to define specific factors required in exocytosis. This model has provided the first evidence of a role for α -SNAP in regulated exocytosis in endocrine cells. This adds to the previously identified factors involved in secretion of insulin, i.e. VAMP-2 and SNAP-25 [12,13]. It seems therefore that important elements of the exocytotic machinery described in neurons [40] are conserved in endocrine cells.

We are grateful to Dr. J. E. Rothman for the gift of plasmids encoding α -SNAP, to D. Nappey and M. Bianchi for technical help and to Dr. K. Sadoul and Dr. R. Regazzi

for useful discussions. This work was supported by the Swiss National Science Foundation grant no. 32-32376.91. C.E.K.-B. is the recipient of an M.D./Ph.D. fellowship from the Swiss Academy of Sciences.

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