Estrogen reduces the risk of Alzheimer's disease in post-menopausal women, β-amyloid (Aβ) burden in animal models of Alzheimer's disease, and secretion of Aβ from neuronal cultures. The biological basis for these effects remains unknown. Here, utilizing cell-free systems derived from both neuroblastoma cells and primary neurons, we demonstrate that 17β-estradiol (17β-E2) stimulates formation of vesicles containing the β-amyloid precursor protein (βAPP) from the trans-Golgi network (TGN). Accelerated βAPP trafficking precludes maximal Aβ generation within the TGN. 17β-E2 appears to modulate TGN phospholipid levels, particularly those of phosphatidylinositol, and to recruit soluble trafficking factors, such as Rab11, to the TGN. Together, these results suggest that estrogen may exert its anti-Aβ effects by regulating βAPP trafficking within the late secretory pathway. These results suggest a novel mechanism through which 17β-E2 may act in estrogen-responsive tissues and illustrate how altering the kinetics of the transport of a protein can influence its metabolic fate.

Reports that post-menopausal estrogen replacement therapy (ERT) is clinically efficacious in delaying the onset of Alzheimer's disease (AD) (1) or improving cognition (2) in post-menopausal women have been corroborated by numerous studies (3–5). Reports questioning the validity of these retrospective studies (6) demonstrated that women with clinically defined AD have no improvement on specific cognitive tasks following short (2–15-month) trials of estrogen treatment. However, the neuroprotective effects of estrogen are hypothesized to safeguard against the development of AD and not to aid in recovering lost function. The biological mechanism(s) through which estrogen exerts its neuroprotective effects remain largely un

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‡ The abbreviations used are: ERT, estrogen replacement therapy; AD, Alzheimer's disease; Aβ, -amyloid; 17β-E2, 17β-estradiol; βAPP, -amyloid precursor protein; TGN, trans-Golgi network; PI, phosphatidylinositol; PITP, PI-transfer protein; PBS, phosphate-buffered saline; ERS, energy-regenerating system; Tricine; N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; WT, wild type; PS1, presenilin 1.
kinases, phospholipase C, and PI-directed phospholipase D (22). These enzymes produce soluble inositol polyphosphates that in turn act as second messengers. These reactions also alter the levels of specific phosphoinositides. Together these effects organize vesicular traffic both spatially and temporally.

Estrogen has also been suggested to alter late secretory pathway vesicle formation. Specifically, electron microscopy in neuroendocrine cells demonstrated an increase in secretory vesicle formation after exposure to estrogen (23). In addition, other steroid molecules such as glucocorticoids have been suggested to regulate trafficking of vesicles from the TGN to the plasma membrane (24, 25). Despite this evidence, a direct link between estrogen and known mechanisms involved in vesicle formation has not been shown. However, studies (26–28) have suggested promising directions for further exploring the link between estrogen and secretory pathway trafficking. For example, transcriptions of methylating enzymes involved in the conversion of phospholipids, and Rab11, a small GTPase involved in the targeting of transport vesicles to the plasma membrane, are up-regulated in response to estrogen (26–28).

In the present study we demonstrate using a cell-free budding assay that estrogen stimulates the budding of βAPP-containing vesicles from the TGN. This alteration in βAPP trafficking leads to a significant decrease in Aβ generation. In addition, we examine the cell biological mechanism underlying this clinically relevant observation. First, we demonstrate that estrogen exposure initiates the recruitment of Rab11 from the cytosol to the TGN. In addition, expressing a C-terminally truncated mutant Rab11 that does not interact with membranes leads to increased Aβ formation, an effect attenuated by 17β-E2. These results suggest a mechanism in which increased Rab11 recruitment to TGN membranes helps initiate budding of vesicles containing βAPP. A separate series of experiments revealed that estrogen also increases the level of PI in the TGN. Furthermore, cell-free assays performed with yeast cytosol containing mutant PITP, a potent PI regulator, corroborate that PI effects organize vesicular traffic both spatially and temporally. Specifically, electron microscopy in neuronal monolayers (9) indicated that estrogen stimulates TGN vesicle formation has not been shown. However, studies (26–28) have suggested promising directions for further exploring the link between estrogen and secretory pathway trafficking. For example, transcriptions of methylating enzymes involved in the conversion of phospholipids, and Rab11, a small GTPase involved in the targeting of transport vesicles to the plasma membrane, are up-regulated in response to estrogen (26–28).

Preparation of Permeabilized N2a Cells—It has been well established that incubation of cells at 20 °C leads to an accumulation of membrane and secretory proteins in the TGN (15, 17). To assay Aβ generation, cells were pulse-labeled with [35S]methionine (500 μCi/ml) for 15 min at 37 °C, washed with PBS (pH 7.4, 140 mM NaCl, 5 mM KCl, 10 mM Hepes, pH 7.2) for 10 min. The buffer was aspirated and replaced with 1 ml of “breaking buffer” (90 mM KCl, 10 mM Hepes, pH 7.2), after which the cells were broken by scraping with a rubber policeman. The centrifuged pellet was homogenized in 800 μl of 1.2 M sucrose, 20% Tricine gels (for Aβ), and 20% Tricine gels (for total protein) by the method of Bradford. Aβ was assayed by running fractions on 4–12% SDS-PAGE, transferring the proteins onto polyvinylidene difluoride membranes, and performing Western blot analysis with 4G8, FCA3440, and FCA3642.

Formation of Nascent Secretory Vesicles in Permeabilized Cells—Following incubation of broken cells, vesicle and membrane fractions were separated by centrifugation at 14,000 rpm for 15 s at 4 °C in a Beckman SW41Ti rotor. Twelve 1-ml fractions were collected from the top of each gradient and assayed for total protein by the method of Bradford. Aβ was assayed by running fractions on 4–12% SDS-PAGE, transferring the proteins onto polyvinylidene difluoride membranes, and performing Western blot analysis with 4G8, FCA3440, and FCA3642.
rpm in 0.25% sucrose for 1 h at 4 °C. Membranes were resuspended in 300 μl containing 2.5 mM MgCl₂, 0.5 mM CaCl₂, 110 mM KCl, and an ERS consisting of 1 mM ATP, 0.02 mM GTP, 10 mM creatine phosphate, 80 μg/ml creatine phosphokinase, and a protease inhibitor mixture. Incubations were carried out at 20 or 37 °C as indicated. Vesicles were isolated and proteins analyzed as described above. Each experiment was performed at least 3 times.

Preparation of Cytosol Fraction from Yeast Secretory Mutants—Cells were grown at 24 °C in yeast extract, peptone, and dextrose (YPD) medium to early log phase. 200 A₅₅₀ units were centrifuged for 5 min at 2500 rpm. Cells were washed twice by dilution in reaction buffer (25 mM Hepes-KOH, pH 7.2, 125 mM KOAc) and once in homogenization buffer (reaction buffer plus 0.5 mM phenylmethylsulfonyl fluoride and 1 mM DTG). 400 μl of acid-washed glass beads were added, and cells were lysed for 8–10 s of agitation in a vortex at full speed. The samples were mixed with 600 μl of homogenization buffer and vortexed four times, each for 30 s at full speed, followed by centrifugation at 3000 × g for 5 min at 4 °C. After transferring to Eppendorf tubes, the supernatant was further centrifuged at 100,000 × g for 1 h at 4 °C. 50-μl aliquots were frozen in liquid nitrogen after protein was measured by the Bradford assay and stored at −80 °C.

Preparation of Mammalian Cytosol Fraction—Ten plates of cells were collected in their media and washed with TEA buffer (10 mM triethanolamine, 140 mM KOAc, pH 7.2) followed by a wash in homogenization buffer (25 mM Hepes-KOH, pH 7.2, 125 mM KOAc, protease inhibitor mixture) and resuspension in a volume of pellet/5 volumes of homogenization buffer. Cells were broken in a Ballet homogenizer (clearance 18 μm) and a post-nuclear supernatant created by centrifuging at 800 × g for 5 min at 4 °C. The post-nuclear supernatant was then centrifuged at 100,000 × g for 1 h at 4 °C. 50-μl aliquots were frozen in liquid nitrogen after protein was measured by the Bradford assay and stored at −80 °C.

Lipid Extraction—Fractions from the sucrose gradients were diluted to 1-ml tubes, transferred to 13 × 100 borosilicate test tubes, and mixed with 3.25 ml of chloroform/methanol 1:2.2 (29). The samples were vortexed and centrifuged to pellet proteins. Supernatants were transferred to clean tubes and phase separated by adding 1 ml each of chloroform and 20 mM acetic acid. After aspirating the upper phases, lower phases were reduced in volume by evaporation under nitrogen stream and dried in a Speed-Vac concentrator.

Phospholipid Analysis—For phospholipid analysis, samples were diluted with 30 μl of chloroform/methanol (2:1), and 10-μl aliquots from each were spotted onto a TLC plate. Plates were developed in chloroform/ethanol/triethylamine/water (30:35:35:7) and visualized by spraying with 3% copper(II) acetate in 8% phosphoric acid followed by charring. Quantitation for phospholipids was performed on a Storm 860 PhosphorImager in the blue fluorescence mode.

Inositide Lipid Extraction and Analysis—The method of Pike and Eakes (30) was used with minor modifications. 1-ml fractions were transferred to 13 × 100-mm borosilicate tubes containing 1 ml of methanol/concentrated HCl (10:1, v/v) and vortexed with 2 ml of chloroform/methanol (10:9, v/v) and mixed with 3.25 ml of chloroform/methanol 1:2.2 (29). The samples were shaken for 1 week (9). By utilizing a cell-free reconstitution system derived from βAPP-expressing neuroblastoma (N2a) cells, we examined whether these decreases were secondary to alterations in βAPP trafficking. The cell-free system has been used extensively as a vehicle within which βAPP trafficking and Aβ generation have been studied (15, 17). In addition, it has been characterized within other cell types (31–33) where electron microscopy has demonstrated the integrity of TGN stacks and TGN budding (31).

N2a cells treated without or with 200 nm 17β-E2 for 1 week were labeled with [3H]methionine and incubated at 20 °C to accumulate βAPP in the TGN. Cells were permeabilized and returned to 37 °C. Cell-free reconstitution of βAPP-containing vesicle budding was followed by centrifugation at 4 °C to halt further βAPP trafficking and processing and to separate nascent vesicles from donor membranes (see “Materials and Methods” for details).

βAPP was isolated by membrane solubilization, immunoprecipitation using a C-terminal-specific antibody (369), and separation using SDS-PAGE. A minor βAPP band is sometimes detected, which may be attributable to incomplete maturation of βAPP. At 20 °C, it is possible that glycosylation is delayed or inhibited. To test this, samples were treated with endo-β-N-acetylgalcosaminidase H and were found to be largely resistant to proteolysis (data not shown) suggesting that immature βAPP accounts for only a small percentage of total βAPP. In addition, it is possible that this band is partially accounted for by endogenous mouse βAPP. βAPP recovered from TGN-derived vesicles after a 90-min in vitro incubation represents 14% of the original pool of labeled βAPP within the TGN. Performing the assay at 20 °C, or omitting the ERS reduces the percentage of βAPP recovered from TGN-derived vesicles to 3.5 and 7%, respectively, of total labeled βAPP (Fig. 1, A and B).

When cell-free preparations are created from cells treated for 1 week with 200 nm 17β-E2, βAPP recovered from the TGN vesicles represents 30% of the labeled βAPP protein within the TGN (Fig. 1, A and B). This increase in budding suggests that 17β-E2 exposure alters cellular trafficking machinery to increase the basal rate of TGN vesicle formation. This increase in TGN vesicle biogenesis could be explained by either activation of integral TGN membrane proteins or changes in the phospholipid composition of the TGN membrane itself. The change cannot, however, be attributed to regulation or activation of cytosolic trafficking proteins because of their removal using extensive washes prior to in vitro incubations.

By stimulating basal TGN budding machinery, 17β-E2 should increase not only the kinetics of βAPP trafficking but that of other proteins within the same subcellular distribution. To assess the kinetics of estrogen-enhanced βAPP budding, [3H]methionine-labeled βAPP-containing TGN membranes, derived from untreated and estrogen-treated cells, were incubated at 37 °C for 30–120 min. The budding of TGN-derived vesicles was increased by prior estrogen exposure at all time points, reaching a maximum at 90 min (Fig. 1, C and D).

Identical experiments were performed studying the trafficking of APLP2, a βAPP-related protein, and TrkB, an unrelated integral membrane protein. TGN budding of both proteins was similarly increased after estrogen exposure for 7 days (data not shown). These results suggest that prior exposure to estrogen results in a consistent and nonspecific increase in the formation of TGN vesicles.

To determine whether the effect of 17β-E2 on βAPP trafficking was reflected in the number of βAPP molecules on the plasma membrane, we labeled cells with biotin for 4 h at 4 °C. Prior estrogen treatment resulted in an increase in βAPP molecules at the cell surface of ~75% (Fig. 1E). Further experiments are underway to assess if estrogen has any effect on the endocytosis of βAPP at the cell surface. It is well accepted that a population of Aβ is generated from full-length βAPP, which has been internalized from the cell surface (34), and future experiments will assess the contribution of estrogen to regulating this segment of βAPP transport. Regardless of the role of 17β-E2 in endocytosis, estrogen-stimulated TGN budding is
Estrogen Stimulates TGN Vesicle Biogenesis

Fig. 1. Estrogen stimulates cell-free formation of post-TGN vesicles containing βAPP. A, N2a cells were labeled with [35S]methionine at 37°C for 15 min followed by a 2-h chase at 20°C to accumulate βAPP in the TGN. Cells were permeabilized and incubated (see "Materials and Methods") for 90 min at 37°C in the presence of an ERS. After incubation, samples were centrifuged, and pellets (lanes 1-4) and supernatants (lanes 5-8) were immunoprecipitated with polyclonal antibody 369 and separated by SDS-PAGE (4-12%). Control incubation conditions include 20°C (lane 5), absence of an ERS (lane 6), and membranes isolated from cells without prior estrogen exposure (lane 7). B, relative βAPP budding following cell-free formation of post-TGN vesicles. Lanes correspond with conditions from lanes 5-8 in A. Values are means ± S.D. from five replications. C, supernatant-derived βAPP following cell-free formation of post-TGN vesicles ranging from 0 to 120 min. TGN membranes were derived from cells incubated in the presence or absence of estrogen prior to experiment. D, βAPP budding after 0-120 min from TGN membranes derived from cells without or with prior estrogen exposure. Values are means ± S.D. from three replications. E, biotinylation of N2a cells incubated either in the absence or presence of estrogen prior to experiment. Biotinylated βAPP (top panel) and total cellular βAPP (bottom panel) was immunoprecipitated using 369 and analyzed by Western blot.

particularly relevant to Alzheimer’s disease pathogenesis, because of the localization of βAPP and Aβ generation within the TGN. These findings raised the possibility that changing βAPP trafficking may result in a direct alteration in Aβ generation.

Stimulation of βAPP-containing vesicle formation from the TGN by estrogen was concentration-dependent, with a minimal effective concentration of 20 nM and a half-maximal effect at 30-50 nM (Fig. 2A). To obtain a maximal effect, 200 nM 17β-E2 was used in the subsequent studies. Interestingly, the effect of 17β-E2 on βAPP trafficking was largely reversed by 50 nM tamoxifen, the nonsteroidal anti-estrogen that blocks estrogen binding to it receptors, although 50 nM tamoxifen alone was able to partially stimulate βAPP budding from the TGN. In addition, 17α-estradiol (17α-E2) also mimics the effect of 17β-E2, however to a lesser extent (Fig. 2B). Together, these data suggest that the specific effect of estrogen on βAPP trafficking is probably mediated by estrogen receptors although non-ER-mediated mechanisms may not be excluded, especially given the 17α-E2 effect. Further investigations are necessary for the elucidation of detailed mechanisms underlying the effect of estrogen on protein trafficking.

Estrogen Reduces Aβ Generation in the trans-Golgi Network—Aβ peptides are generated from βAPP with variable N and C termini. The ~4-kDa peptide can end at either amino acid 40 (Aβ40) or amino acid 42 (Aβ42). Although the endoplasmic reticulum has recently been implicated in the generation of specific insoluble and non-secreted Aβ peptides, the major site of generation for secreted Aβ is the TGN (17).

To examine whether estrogen’s stimulation of βAPP trafficking from the TGN altered Aβ generation, βAPP-transfected N2a cells were treated without or with 200 nM 17β-E2 for 1 week, labeled with [35S]methionine for 3 h, and homogenized in 0.25 M sucrose. Cellular extracts were separated by centrifugation using a discontinuous sucrose gradient, and Aβ peptides were immunoprecipitated from sequential fractions using antibody 4G8. The gradient distribution of Aβ levels in all Aβ-containing TGN fractions following estrogen treatment, but no apparent redistribution (Fig. 3A), demonstrating that in intact cells the subcellular localization of Aβ peptides is unaffected by estrogen treatment, whereas its generation appears to be significantly reduced.

To examine the effect of estrogen treatment upon the de novo generation of Aβ within the TGN, cell-free assays were performed. Aβ peptides were immunoprecipitated with antibodies specific for the two major C termini (40/42). In the presence of an ERS at 37°C, Aβ40 and Aβ42 were both detected within the TGN at 37°C (Fig. 3B, lanes 1 and 5). When cell-free TGN preparations were generated from cells treated for 1 week with estrogen there was a 37% reduction in Aβ40 formation and an 83% reduction in Aβ42 formation (Fig. 3B, lanes 2 and 6). Within post-TGN vesicles, 17% of total Aβ40 and 14% of total Aβ42 were recovered (Fig. 3B, lanes 3 and 7). Those levels were further reduced following 17β-E2 treatment (Fig. 3B, lanes 4 and 8).

Despite quantitatively less Aβ42 production in the estrogen-treated cells lines, both Aβ40 and Aβ42 were transported at equal rates (22% versus 19%), suggesting that the major effect of estrogen on Aβ metabolism is not on Aβ trafficking but rather on de novo TGN Aβ generation. That estrogen might regulate the trafficking of βAPP, but not that of its metabolite Aβ, is plausible; βAPP is an integral plasma membrane protein whose cell surface residence and therefore its TGN packaging and transport should be closely regulated. Aβ variants, as by-products of aberrant βAPP proteolysis, are more likely pack-
immunoprecipitated using antibody 4G8 and separated using 4–/H9252 termi-

de whether full-length cell-surface /H9252 tion demonstrates that

in the legend to Fig. 1 except that supernatants were sequentially immunopre-

combination of 200 nM 17

of the effect of estrogen on formation

A

aged into post-TGN vesicles within anterograde bulk flow.

Endocytosis is not excluded as a relevant pathway that may

count to Aβ generation. Data from cell-surface biotinyla-

demonstrates that βAPP increases with estrogen. To de-

terminate whether full-length cell-surface βAPP contributes to

Aβ generation would require budding assays using purified

membrane fractions. Although this question is exceedingly in-
	
teresting, it is well beyond the scope of the present studies and

represents a logical continuation of the project.

Cytosol from Estrogen-treated Cells Stimulates Cell-free TGN

Budding—The importance of cytosolic trafficking proteins in

the genesis and budding of TGN vesicles is well established

(reviewed in Ref. 35). It is not known, however, whether these

trafficking factors are regulated by estrogen. To study the

effects of estrogen upon cytosolic regulation of TGN vesicle

biogenesis, we utilized the cell-free trafficking assay.

Cytosol prepared from N2a cells expressing human βAPP695 were incubated for 7 days in the

absence or presence of various concentrations of 17β-E2 (A), 200 nm 17α-E2, or a

combination of 200 nm 17β-E2 and 200 nm tamoxifen (Tam) (B). Standard cell-free

vesicle budding assays were performed as described in Fig. 1. Representative autoradiographic and quantitative analyses of

formation of βAPP-containing TGN vesicles are shown. Data represent means ±

SD; n = 3.

and nearly 2.5× when compared with identical reactions with-

out cytosol (Fig. 4B, lanes 3 versus 7).

We also examined whether estrogen-primed cytosol would

have any effect on TGN budding in cells already exposed to

estrogen. Cell-free assay systems prepared from 17β-E2-
treated cells were incubated at 37°C without cytosol, with

estrogen-naive cytosol, or with estrogen-primed cytosol. In the

absence of any exogenous cytosol, TGN budding was stimulated

2× in cells that had been pre-treated with 17β-E2 (Fig. 4B, lanes 3 versus 4). Addition of estrogen-naive cytosol only

slightly increased TGN budding in both cell conditions (Fig. 4B, lanes 3 and 4 versus 5 and 6). Addition of estrogen-primed cytosol to untreated cells significantly increased βAPP budding when compared with estrogen-naive cytosol (Fig. 4B, lanes 5 versus 7) but did not have an additive effect upon the mem-

branes derived from 17β-E2-pretreated cells (Fig. 4B, lanes 6 versus 8). These results suggest that treating cells with 17β-E2 for 1 week generated a maximally active secretory pathway that could not be further stimulated.

Estrogen Stimulates TGN Budding in Neuronal Cell-free Systems—We examined whether this stimulatory effect was

limited to cytosol derived from N2a cells. We prepared cytosol from human, rat, and mouse primary fetal cortical neurons that had been untreated or treated with 200 nm 17β-E2 for 1 week. A greater than 2-fold increase in TGN budding was demonstrated in all three species examined, comparing estro-
gen-naive versus estrogen-primed cytosol (Fig. 4C). That human, rat, and mouse estrogen-primed cytosol could stimulate TGN budding suggested that neuronal cytosol could be used to identify estrogen-responsive factors and that mammalian neurons may also be used directly for in vitro assays.

To explore this possibility, we established a cell-free reconstitution system using primary neuronal cultures (see “Materials and Methods”). Budding of βAPP-containing vesicles was increased from TGN membranes derived from estrogen-treated rat and mouse neurons when compared with TGN membranes derived from untreated neuronal cultures (Fig. 4D). Thus primary neuronal cultures appear to be influenced by chronic estrogen administration in a fashion similar to that of N2a cells, suggesting that common trafficking mechanisms are employed in neurons and neuronal cell lines.

**Estrogen Recruits Rab11 to TGN Membranes**—Rab11, a cytosolic GTPase, is important for both formation of TGN-derived vesicles and for vesicle formation during endocytosis (36, 37). We studied Rab11 because of a report suggesting that Rab11 is induced in response to estrogen administration (26). We found that Rab11 protein levels were not significantly changed in mouse N2a cells following 1 week of estrogen exposure (Fig. 5A). Cytosolic Rab11 protein levels were slightly decreased following estrogen treatment in both mouse N2a cells (Fig. 5B) and human primary neuronal cultures (data not shown).

When protein levels were assayed following subcellular fractionation, Rab11 was highly enriched in the TGN and endosomal membrane fractions derived from estrogen-treated cells (Fig. 5D). These results suggest that estrogen stimulates the recruitment of cytosolic Rab11 to the TGN membranes. Although the change of Rab11 localization in the presence of estrogen may be significant, it seems likely that Rab11 will be only one of many proteins, working in a complex, to initiate changes in vesicle traffic originating from the TGN. Interestingly, it has been reported that Rab11 forms an insoluble complex with presenilin 1 (PS1) (38), a protein mutation of which causes the majority of early onset forms of AD. Future studies will be aimed at determining whether PS1 mutations
Estrogen Stimulates TGN Vesicle Biogenesis

FIG. 6. Rab11ΔC induces increases in Aβ production that are attenuated by 17β-E2. A, doubly transfected cells expressing βAPP<sub>695</sub> and either wild-type Rab11 (Rab11WT) (lane 1) or a C-terminally truncated Rab11 (Rab11ΔC) (lane 2) expressed equal levels of full-length βAPP. B, both cells lines were incubated for 1 week in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 200 nM 17β-E2. Cells were continuously labeled for 4 h with 500 μCi/ml [35S]methionine. Intracellular (upper panel) proteins were extracted, and secreted (lower panel) proteins were collected. Proteins were immunoprecipitated by 4G8 and subjected to Tricine SDS-PAGE (10–20%) analysis.

alter late secretory pathway traffic either dependent on, or independent of, Rab11 involvement.

That estrogen influenced both βAPP and Rab11 localization suggested a possible link between the trafficking factor and the transport of βAPP. Utilizing doubly transfected cells expressing βAPP<sub>695</sub> and either wild-type Rab11 (Rab11WT) or a C-terminally truncated Rab11 (Rab11ΔC) which cannot interact with membranes, we examined if a mutated Rab11 would disrupt βAPP trafficking and therefore TGN Aβ production.

Despite equal levels of βAPP expression in the two cells lines (Fig. 6A), βAPP/Rab11ΔC cells produced significantly more TGN Aβ and secreted more Aβ than control cells expressing βAPP/Rab11WT (Fig. 6B, lane 3 versus 1), suggesting that βAPP trafficking was likely altered. Prior treatment of these cell lines with estrogen attenuated these alterations in Aβ production (Fig. 6B, lanes 1 and 3 versus 2 and 4). These results suggest that Rab11ΔC limits basal trafficking by restricting TGN vesicle formation. This would reduce budding of βAPP and lead to increased Aβ formation.

Estrogen may be acting to increase basal budding through multiple routes. One possibility is through recruiting endogenous WT Rab11. Transfected Rab11ΔC may compete with WT Rab11 for binding with other necessary trafficking factors to influence budding. Alterations in membrane lipid composition also could contribute to the demonstrated change in budding. Data in support of the second model are presented below.

Estrogen Alters TGN Phospholipid Composition—Protein trafficking within the secretory pathway is believed to be due to a combination of events. The recruitment of cytosolic proteins to the membranes and inherent changes in the lipid composition are believed to initiate the curvature of the membranes (39). That TGN membranes were shown to have a higher rate of vesicle formation after estrogen treatment, in the absence of cytosol, suggested that inherent membrane rearrangements must, in part, contribute to this enhanced vesicle biogenesis. To examine if estrogen influences TGN membrane composition, we measured TGN phospholipid levels using subcellular fractionation and TLC.

The TLC analysis revealed a slight loss of phosphatidyethanolamine, phosphatidylserine, and phosphatidylcholine in TGN membranes after estrogen treatment. At the same time, an increase in these lipids is seen in the post-TGN vesicles with estrogen treatment (Fig. 7, A and B). Because the regulation of phosphatidylinositol (PI) levels by PI-transfer protein (PITP) is essential in membrane vesiculation (40), we examined PI levels in TGN and post-TGN vesicles following estrogen exposure. PI incorporation into TGN membranes and subsequently formed TGN vesicles is increased with estrogen (Fig. 7, C and D). More importantly, the PI shifted from the TGN to the vesicle fraction in estrogen-treated cells. These data suggest that one mechanism through which estrogen stimulates vesicle biogenesis and βAPP trafficking is through a cytosolic factor-independent redistribution of PI from TGN membranes into nascent vesicles.

The increased phospholipid mass in vesicular fractions following estrogen treatment is likely a result of increased vesicle numbers, not an alteration of vesicle composition; these lipids are most likely structural components of the vesicles, not necessarily part of the budding machinery. Increasing TGN vesicle formation does not necessarily require an increase in total lipid synthesis. Increased vesiculation could be caused by a net shift of lipid mass from the TGN to transport vesicles resulting in an increase in the number of vesicles without altering the phospholipid composition of these vesicles.

Mutations in PITP Inhibit TGN Budding—To study the effects of altering phospholipid levels on TGN budding, we utilized the fact that mammalian neurons and the budding yeast Saccharomyces cerevisiae are highly homologous in many of the proteins regulating secretion. Temperature-sensitive mutants of S. cerevisiae block specific points in the secretory pathway at non-permissive temperatures (reviewed in Ref. 41). Cytosol from WT or mutant yeast strains was used to study TGN budding in the N2a cell-free budding assay. Cytosol from WT, Sec1 (yeast homologue of mammalian Munc-18), and Sec18 (yeast homologue of mammalian NSF) mutant cells supported budding (Fig. 8, lanes 2–4 and 6). In contrast, cytosol from Sec14 (yeast homologue of mammalian PITP) reduced budding to 18% of control (Fig. 8, lane 5).

The inability of cytosol containing mutant Sec14 to support TGN budding is consistent with the function of its mammalian homologue PITP. Mutations in Sec14 or PITP severely restrict the ability of cells to form new secretory vesicles from the TGN (42). These results support the role of phosphatidylinositol in the budding of nascent vesicles from the TGN, a phenomenon demonstrated to be estrogen-responsive. Future studies will be needed to determine which other TGN budding proteins in addition to Rab11 may be responsive to estrogen.

DISCUSSION

The ability of estrogen replacement therapy to delay the onset of AD in post-menopausal women raised speculation as to the mechanism through which it exerted these protective effects. Estrogen has long been viewed as a neuroprotective molecule. Thus it was unclear whether the ability of estrogen to delay AD onset was due to specific amelioration of an AD-associated pathology or a nonspecific neuroprotective response. The demonstration by Xu et al. (9) that the secretion of β-amyloid peptides, a central and unvarying component of AD pathology, was reduced in cells incubated in the presence of estrogen, suggested a direct influence of estrogen upon βAPP metabolism.

Although estrogen has myriad effects within cells, a direct influence upon discrete components of secretory pathway machinery has not been reported. Several studies reporting effects upon the morphology of individual compartments within the secretory pathway suggested that protein transit though those compartments could be influenced by estrogen. In particular, the TGN appears to vesiculate in response to long term exposure to estrogen. That βAPP trafficking through the TGN may be accelerated by estrogen suggests a direct mechanism by which estrogen alters levels of Aβ secretion; by decreasing the...
Estrogen Stimulates TGN Vesicle Biogenesis

Fig. 7. Phosphatidylinositol is redistributed from TGN to post-TGN vesicles in response to 17β-E2. A, vesicle and TGN fractions were prepared using subcellular fractionation of N2a cells incubated in either the absence or presence of 17β-E2. Phospholipids were extracted from these fractions and analyzed using TLC as described under "Materials and Methods." B, quantification of TLC represented as % change in TGN or vesicle fractions after 17β-E2 treatment. C, vesicle and TGN fractions were prepared as described above, except that N2a cells were labeled first with tritiated inositol as described under "Materials and Methods." D, quantification of tritiated phosphatidylinositols before and after 17β-E2 treatment in vesicle and TGN fractions. PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PIP₂, phosphatidylinositol bisphosphate.

The pharmacological studies of the estrogen concentration dependence were necessary to assess if the effect of estrogen on βAPP metabolism and trafficking was physiologically relevant. Previous in vivo animal studies demonstrated estrogen concentration dependence of Aβ formation similar to studies in whole cells. Here we demonstrate that cell-free Aβ generation/secretion is also dose-dependent, consistent with those studies in whole cells and animal models.

To elucidate which pathway estrogen is exerting these βAPP trafficking and Aβ-lowering effects, we studied the effects of 17α-E2, as well as those of tamoxifen in identical cell-free βAPP trafficking assays to those performed with 17β-E2. Tamoxifen did support βAPP trafficking to a lesser degree but more importantly inhibited the robust effect of 17β-E2 when the two were co-administered. Also, 17α-E2 was surprisingly effective at stimulating βAPP trafficking, although again not quite as efficient as 17β-E2. These results showing that different estrogen analogues support vesicle budding with varying degrees of success suggest that in addition to a likely direct genomic effect of 17β-E2, the indirect genomic effects involving estrogen receptor/mitogen-activated protein kinase/extragranular signal-regulated kinase pathways also probably play a role (7, 43).

There is a significant barrier to a complete understanding of steroid neurochemistry in the brain; this occurs because many actions of estrogen are mediated via different intracellular mechanisms with the same compound acting as an agonist in one type of neuron and an antagonist in a neighboring cell. In addition to different receptor-mediated pathways, a large number of possible non-genomic effects of estrogen, e.g. reducing oxidative insults, cannot be ruled out. It is clear that many additional studies will need to be performed in receptor knockout or kinase-deficient cell lines, if the field is to begin to understand these competing pathways adequately.

The central findings derived from these experiments suggest several immediate productive areas of investigation. The major conclusions include the following: 1) estrogen influences the kinetics of protein transport through the TGN; 2) this alteration significantly influences the metabolism in diminished Aβ production, and enhancement of TGN vesicle biogenesis which occurs from recruitment of Rab11 to the TGN membranes from the cytosol and an alteration of PI within the TGN. It is likely that lipids and proteins other than PI and Rab11 are redistributed following estrogen treatment. Never-
theless, the finding that it is possible to affect the rate of βAPP metabolism by altering its trafficking within neurons by modulating the levels of an endogenously produced steroid provides a plausible mechanism by which estrogen is clinically efficacious at delaying or preventing AD. In addition, this basic conceptual framework raises the possibility that other errors of protein metabolism or transport that occur as a result of somatic disease or simply as a consequence of decreased estrogen production late in life may be similarly rectified by resetting the rate of protein transport within the secretory pathway with estrogen administration.

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