Calcium- and Tyrosine Phosphorylation-dependent Mechanisms of Amyloid Precursor Protein Processing

by

Magdalena A. Petryniak

Submitted to the Department of Electrical Engineering and Computer Science
in Partial Fulfillment of the Requirements for the Degrees of
Bachelor of Science in Electrical Science and Engineering
and Master of Engineering in Electrical Engineering and Computer Science
at the Massachusetts Institute of Technology

May 1995

Author.....
Department of Electrical Engineering and Computer Science

Certified by.....
Richard J. Wurtman, Thesis Supervisor

Accepted by.....
F.R. Morgenthaler
Chairman, Department Committee on Graduate Theses

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ABSTRACT

The formation of amyloidogenic derivatives of the amyloid precursor protein (APP) has been linked to the disruption of neuronal calcium homeostasis in Alzheimer's disease. Muscarinic receptor activation stimulates secretory processing of APP via protein kinase C (PKC) and tyrosine phosphorylation, generating non-amyloidogenic APPs peptides (Nitsch et al., 1992; Slack et al., 1995). Using human embryonic kidney cells (293) transfected with m3 muscarinic receptors, I investigated whether elevations in cytosolic calcium similarly influence APP processing. Calcium influx induced with ionomycin mimicked the effects of muscarinic receptor activation with carbachol on tyrosine phosphorylation and on APPs secretion. These effects of calcium influx were found to be partially independent of PKC. Abolishing calcium influx by chelating with EGTA inhibited carbachol-stimulated tyrosine phosphorylation and APPs secretion. These results indicate that muscarinic receptors regulate APP processing by activating at least two signaling cascades, one dependent on tyrosine phosphorylation secondary to calcium influx and the other on PKC.

Thesis Supervisors: Barbara E. Slack Ph.D., Department of Pathology, Boston University School of Medicine, and Richard J. Wurtman M.D., Department of Brain and Cognitive Science, MIT.

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List of Abbreviations and Definitions

Aβ	β–amyloid peptide
ACh	acetylcholine
APP	amyloid precursor protein
APPs	secreted derivative of amyloid precursor protein
CaM	calmodulin
Carbachol	muscarinic receptor agonist
DAG	diacylglycerol
EGTA	calcium chelator
ER	endoplasmic reticulum
GF109203X	protein kinase C inhibitor
G-protein	guanine nucleotide binding protein
Ionomycin	calcium ionophore
IP_3	inositol trisphosphate
PI	phosphatidylinositol
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate; PKC agonist
ROCC	receptor-operated calcium channel
Thapsigargin	inhibitor of calcium uptake into IP_3-sensitive pools
Tyrphostin A25	tyrosine kinase inhibitor

Chapter 1: Introduction

Alzheimer's disease is a neurodegenerative disorder characterized by memory loss and dementia. The pathological markers of the disease include loss of cortical cholinergic innervation, neurofibrillary tangles, and extensive deposition of amyloid plaques. The core component of amyloid plaques is the peptide Aβ, a derivative of a transmembrane glycoprotein termed the amyloid precursor protein (APP). A fraction of the cellular APP pool is cleaved in manner which precludes the formation of Aβ and results in secretion of its N-terminal fragment (APPs) into the extracellular medium. It has been demonstrated that the secretory, non-amyloidogenic degradation of APPs may be enhanced by activation of cell-surface receptors linked to phosphoinositide hydrolysis, and subsequent production of second messengers. However, the cellular signaling mechanisms by which neurotransmitter activation of receptors results in non-pathologic APP processing are not yet fully understood.

Recent studies have implicated protein kinase C (PKC) activation as well as elevations in cellular calcium and tyrosine phosphorylation in the regulation of APP processing by muscarinic receptors coupled to phosphoinositide breakdown. Buxbaum and colleagues (1994) showed that secretory processing of APP is mediated by IP_3-induced calcium release from internal stores following receptor-initiated phosphoinositide (PI) hydrolysis. They hypothesized that two cellular signaling cascades, one involving PKC and the other calcium, contribute to APPs cleavage. The findings of Slack et al. (1995) indicate that stimulation of APPs secretion by muscarinic receptor activation is mediated by tyrosine phosphorylation in a manner partially independent of PKC. However, the interactions between these signal transduction events have not been elucidated. Furthermore, a study by Querfurth et al. (1994) positively correlated calcium influx with increased Aβ

production, but did not exhaustively address its effects on APPs secretion. Thus, the possibility that calcium influx elicited by muscarinic receptor activation culminates in APPs release remains to be investigated.

The goal of this study is to demonstrate that calcium influx secondary to muscarinic receptor activation stimulates APPs secretion by a mechanism dependent on tyrosine phosphorylation. I begin by showing that calcium influx induced with ionophores affects APPs secretion similarly to muscarinic receptor agonists. Next, I address the question of what signaling events, PKC activation, tyrosine phosphorylation, or both, mediate the effects of calcium on APPs release. I conclude by discussing the evidence for multiple cellular signaling cascades recruited by muscarinic receptors to stimulate secretory processing of APP.

Chapter 2: Background

2.1. Overview of Signaling Pathways Involved in APP Processing

One of the hallmarks of Alzheimer's disease pathology is the presence of senile plaques in the brains of patients with the disease. These plaques are composed of aggregated peptides (Aβ) derived from a larger parent protein termed the amyloid precursor protein (APP). APP is a transmembrane protein with a large extracellular domain that is expressed in a variety of cell types (1). Under normal conditions APP is cleaved at an extracellular site within the Aβ domain by an uncharacterized enzyme called α-secretase (2). This secretory processing event leads to the release of a large soluble fragment of APP (APPs) into the interstitial medium and precludes the formation of Aβ (2). Alternatively, APP may be cleaved in intracellular compartments, giving rise to the intact Aβ fragment which can aggregate into amyloid plaques under certain, undefined conditions (3). Since APPs and Aβ appear to be formed by two mutually exclusive mechanisms (4, 5), stimulating secretory processing of APP may prevent formation of Aβ and its accumulation into amyloid plaques. The therapeutic implications of this possibility are clear. Although other evidence indicates that the release of APPs and Aβ may proceed independently of each other (6), the ability of APPs to protect neurons against excitotoxic and ischemic insults provides an additional rationale for examining the mechanisms regulating its secretion (7).

Release of APPs can be initiated by binding of acetylcholine analogs to muscarinic cell-surface receptors linked to phosphatidylinositol (PI) hydrolysis (8). Stimulation of m1 and m3 muscarinic receptor subtypes leads

to G-protein (guanine nucleotide binding protein)-mediated activation of PI-specific phospholipase C (9). Phospholipase C catalyzes the breakdown of PI to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). The formation of DAG at the cytosolic surface of the plasma membrane, in conjunction with calcium release from internal stores by IP_3, results in activation of protein kinase C (PKC) (10). Direct activation of PKC by phorbol esters, which mimic the effects of DAG, has been shown to cause release of APPs (11) and inhibit production of Aβ (5, 6, 12), suggesting that this enzyme mediates the effects of muscarinic receptor stimulation on APP processing. However, inhibition of PKC with specific antagonists fails to suppress completely the APPs release induced with ligands of muscarinic receptors (13). This evidence indicates that PKC is not the sole mediator of muscarinic effects on APP processing, and that other signaling molecules may also be involved.

Another signaling event which could effect APPs release is protein tyrosine phosphorylation, known to be increased upon activation of muscarinic receptors coupled to G-proteins and PI hydrolysis (13, 14). Increases in tyrosine phosphorylation in cells may result from a rise in activity of tyrosine kinases or a decline in the activity of tyrosine phosphatases (13, 14). Elevations in tyrosine phosphorylation induced by treatment of cells with tyrosine phosphatase inhibitors has been shown to evoke APPs release (13). Moreover, tyrosine kinase inhibitors antagonized the stimulation of APPs release by muscarinic receptor agonists. These findings suggest the existence of multiple regulatory mechanisms that mediate the effects of muscarinic receptor activation on tyrosine phosphorylation and downstream APP processing.

2.2. The Potential Role of Calcium in Regulating APPs Secretion

Calcium is a natural candidate for a messenger in the signaling cascade regulating APP metabolism. It plays a vital role in numerous cellular processes, and its homeostasis is essential for cell survival (7). APPs is involved in stabilizing neuronal calcium concentrations and hence in protecting neurons against excitotoxic or ischemic insults; it is capable of causing a marked and reversible reduction in free cytosolic calcium (7). On the other hand, aggregation of Aβ leads to disruption of neuronal calcium homeostasis, by interfering with membrane proteins regulating calcium, or by forming pores in the plasma membrane resulting in a toxic influx of calcium (7, 15, 16). These differential roles of APPs and Aβ raise the possibility of a feedback mechanism whereby calcium concentration is "sensed" via a calcium-dependent step in the APP regulatory pathway and is able to influence APP processing in favor of APPs production.

There are several points in the signal transduction pathway between PI-coupled muscarinic receptor activation and APP processing at which calcium could come into play. The PI degradation product IP_3 causes a transient rise in cytosolic calcium levels, by releasing calcium from intracellular stores such as the endoplasmic reticulum (ER). The release of calcium may affect APP release via PKC, since the majority of PKC subtypes are dependent on calcium as well as on DAG (10, 17). Furthermore, high concentrations of calcium may activate PKC at smaller concentrations of DAG (17). Evidence has also been presented for PKC-independent stimulation of APPs secretion by IP_3-generated intracellular calcium. In Chinese hamster ovary and neuroglioma cell lines, elevations in intracellular calcium elicited by inhibition of the ER calcium reuptake mechanism with thapsigargin and cyclopiazonic acid led to an increase in APPs release and a concomitant reduction in Aβ; the effects persisted after PKC activity had been downregulated (18).

The cell can also regulate calcium levels via channels in the plasma membrane that permit influx of calcium from the extracellular space. Elevations in cellular calcium due to influx may likewise affect APP processing. Calcium influx associated with cell-surface receptor stimulation has been studied extensively. In numerous cell types, stimulation of PI-coupled cell-surface receptors produces a rapid transient spike in intracellular calcium levels, followed by a lesser but sustained elevation in calcium concentration. The transient rise in calcium is attributed to release from internal stores, as described above, while the sustained increase is the result of calcium entry from the extracellular space (19).

One or more mechanisms may underlie receptor-activated calcium influx. In some cell lines, calcium entry is triggered by depletion of intracellular calcium stores (20). In parotid acinar cells, depletion of intracellular calcium pools with thapsigargin (a compound that inhibits active calcium transport into IP_3-sensitive pools) mimicked the effect of a muscarinic receptor agonist on both the transient and sustained elevations in cytosolic calcium levels (20). In many cell types, calcium enters through receptor-operated calcium channels (ROCCs) upon binding of a ligand to the receptor (19, 21). ROCCs may also be indirectly activated by IP_3, or by intracellular calcium, or may be regulated independently of these second messengers (21). Receptor-operated, IP_3-independent calcium influx has been shown to play a pivotal role in mediating certain functions of m1 and m3 muscarinic receptors (22). Calcium entry elicited either directly or indirectly by muscarinic receptor activation, represents another mechanism by which cholinergic agonists may increase secretory processing of APP.

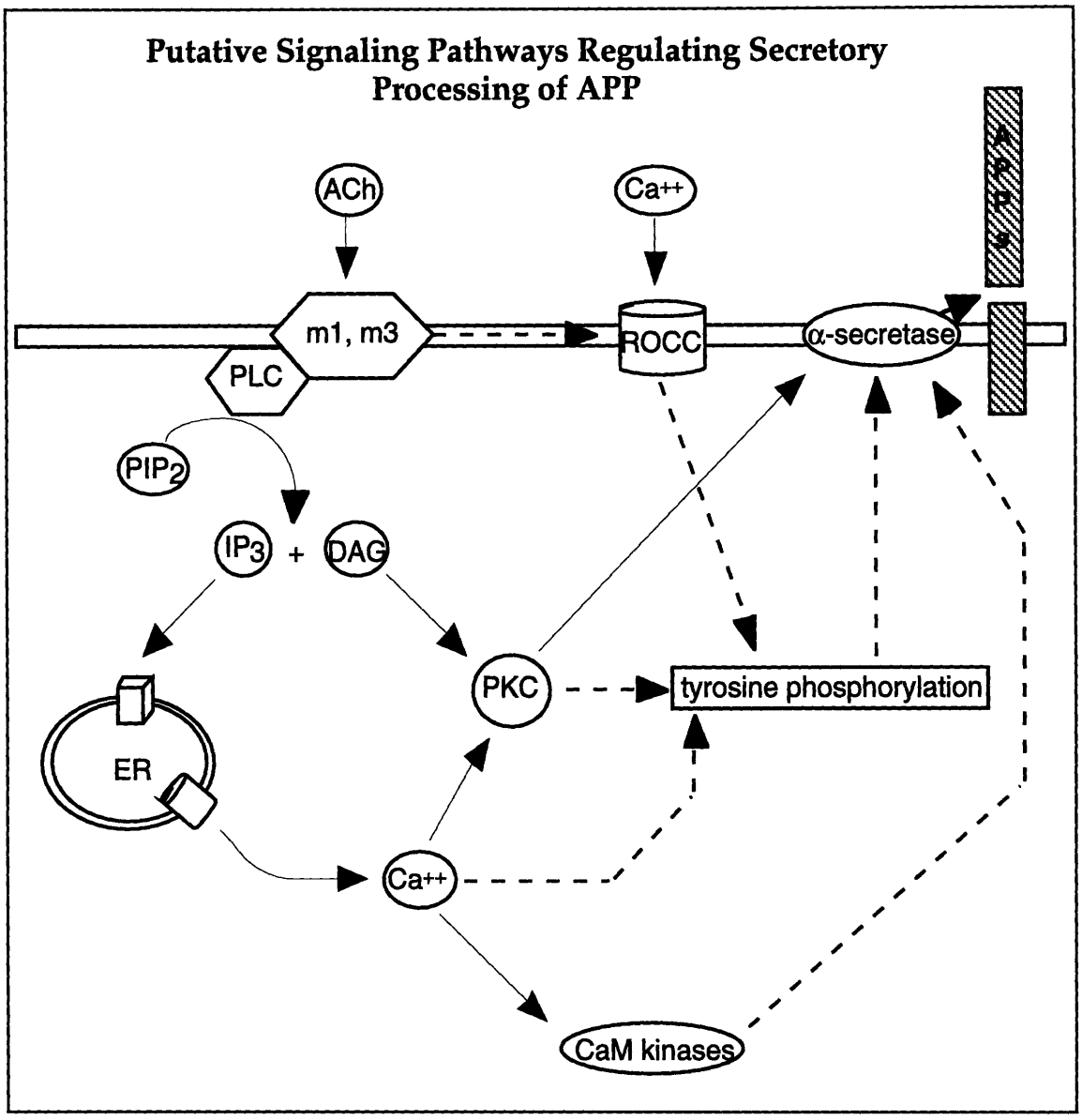
If calcium mediates APPs release following muscarinic receptor stimulation, it is plausible that its effects are due to tyrosine phosphorylation. There is ample evidence illustrating the dependence of tyrosine phosphorylation on calcium. Studies performed in Chinese hamster ovary cells transfected with m5 muscarinic receptors showed that calcium influx is required for stimulation of tyrosine phosphorylation by m5 receptor

activation (22). In hippocampal slices and in neurons in primary culture, depolarization and muscarinic receptor activation induced tyrosine phosphorylation in a calcium-dependent manner (23). Furthermore, ionophore-evoked calcium influx stimulated tyrosine phosphorylation in hippocampal slices, via a mechanism which may be independent of PKC (23).

Additional evidence for PKC-independent calcium-mediated control of tyrosine kinase phosphorylation was obtained from human platelets (24). Inhibition of PKC activity did not abolish tyrosine phosphorylation induced by calcium released from intracellular pools with thapsigargin. However, chelation of extracellular calcium was found to completely abolish tyrosine phosphorylation induced by depleting intracellular pools with thapsigargin. This implicates calcium influx, as opposed to calcium mobilized from intracellular stores, in triggering tyrosine phosphorylation. However, additional data from human platelets suggests that both IP_3-evoked calcium transients and calcium influx could lead to tyrosine phosphorylation (24). Moreover, tyrosine kinase activity may in fact regulate calcium influx stimulated by receptors linked to phosphatidylinositol turnover or by depletion of intracellular calcium stores (25, 26).

The regulatory effects of calcium on APPs secretion may also be mediated by calcium/calmodulin-dependent protein kinases (Ca^{++}/CaM kinases), which have been shown to phosphorylate APP peptides in vitro (27). Ca^{++}/CaM kinase activation and consequent protein phosphorylation may be the result of cytosolic calcium transients generated by IP_3, as has been observed in a variety of endocrine and neural systems (19). Alternatively, tyrosine phosphorylation may modulate the activity of Ca^{++}/CaM kinases (28). The various pathways through which calcium may affect processing of APP are summarized in figure 1.

FIGURE 1



Putative Calcium and Tyrosine Phosphorylation-Dependent Mechanisms of APPs Processing. Activation of m1 or m3 muscarinic receptors by acetylcholine *(ACh)* or its analogs leads to G-protein coupled phosphoinositide *(PIP_2)* breakdown into the second messengers diacylglycerol *(DAG)* and inositol trisphosphate *(IP_3)*. IP_3 releases calcium from intracellular pools, such as the endoplasmic reticulum *(ER)*, while DAG activates protein kinase C, *(PKC)*. Class A PKC isozymes are dependent on calcium for activation. The IP_3-evoked calcium transient may stimulate calcium/calmodulin-dependent protein kinases *(CaM kinases)* which in turn may influence APPs processing. Muscarinic receptor activation may also induce calcium influx, either directly via receptor-operated calcium channels *(ROCC)*, or indirectly via depletion of cellular stores (not shown). These signaling events may converge at the level of tyrosine phosphorylation, which in turn elicits APPs secretion by an unknown mechanism.

Chapter 3. Experimental Procedures

3.1. Cell Culture

Human embryonic kidney (HEK) 293 cell lines transfected with m1 and m3 muscarinic receptor subtypes were used as a model to study of effects of calcium on APPs release. HEK cells were cultured in DMEM/F12 bicarbonate medium containing 10% fetal calf serum, and maintained at 37°C in an atmosphere of 5% CO_2. For experiments, cells were grown to confluency in plastic dishes precoated with poly-D-lysine and grown at the above specified atmospheric conditions. Upon reaching confluency, cells were treated with pharmacological agents diluted to the desired concentrations in serum-free medium, and incubated for appropriate period of time (see 3.2. Pharmacological Agents). Following treatments, media and cell pellets were collected for subsequent analysis.

3.2. Pharmacological Agents

The following pharmacological agents were used to investigate the dependence of APPs secretion and tyrosine phosphorylation on calcium. The muscarinic receptor agonist carbachol was used to elicit APPs release and tyrosine phosphorylation. The phorbol ester PMA (phorbol 12-myristate 13-acetate) was used for activation of PKC. Inhibition of PKC activity was brought about by a PKC-specific blocker, GF109203X. Chelation of extracellular calcium was achieved with EGTA (ethylene glycol-bis-(β-aminoethyl)-N,N,N',N'-tetra aceoxymethyl ester). Calcium influx was stimulated with the calcium ionophore ionomycin. W-7, an antagonist of calmodulin, was used as an inhibitor of calcium/calmodulin-dependent

kinase activity. Tyrphostin A25 was administered as an inhibitor of tyrosine kinase activity. PMA and EGTA were purchased from Sigma (St. Louis, MO); GF109203X from LC laboratories (Wobum, MA), and ionomycin along with W-7 were obtained from Calbiochem (La Jolla, CA).

For treatment preparation, pharmacological substances were diluted to appropriate concentration in serum-free DMEM and preincubated at 37°C in a 5% CO_2 atmosphere. Where necessary, stock solutions were prepared by dissolving compounds in dimethyl sulfoxide, then further diluting in serum-free medium. Prior to application of treatments, cells were washed with 2 ml serum-free DMEM to remove floating cells and debris. For measurements of APPs release, cells were incubated in treatment solutions for a period of 1 hr.[1] For anti-phosphotyrosine immunoprecipitation experiments, cells were incubated with treatments for 10 minutes (the time required to achieve maximal protein phosphorylation (unpublished results)). Experiments involving tyrphostin A25 required preincubation with this substance or a vehicle (dimethyl sulfoxide) control for a period of 18-24 hours prior to administration of acute treatments as described above.

3.3. Measurement of APPs Release[2]

Media Preparation. Following treatment incubation, media were removed from cell dishes, centrifuged for 2 minutes to remove debris, mixed with 40 µl of the protease inhibitor PMSF (Sigma), and placed on ice. Media were desalted and dried overnight. Media residues were subsequently diluted 1:1 in extraction buffer containing 2% Triton X-100 and 2% Nonidet P-40 and gel loading buffer.

Pellet Preparation. Cells were rinsed with 2 ml phosphate-buffered saline (PBS), harvested in 1 ml PBS, mixed with 20 µl PMSF, and placed on ice. Cell

[1] Maximal APPs release is reached within 30min, and remains stable for 60 min (Nitsch et al., 1992).

suspensions were subsequently centrifuged, the supernatant removed, and the cell pellets were lysed in the extraction buffer used for media samples. Lysates were then centrifuged to remove detergent-insoluble material and suspended 1:1 in gel loading buffer.

Electrophoresis and Western Blotting. Media and pellet samples were boiled for 2 min to denature proteins, and uniform amounts of each sample (the equivalent of 300 μg or 400 μg of cell protein for media and 150 μg or 200 μg of cell protein for pellet; loading volume was determined using the bicinchoninic acid protein assay) were loaded into 12% mini-gels (Bio-Rad, Richmond, CA). Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). At the conclusion of electrophoresis proteins were electroblotted onto polyvinylidene difluoride membranes and blocked for 30 minutes with 5% powered milk in Tris-buffered saline with .15% Tween 20 (TBST). Subsequently, membranes were immunoblotted with anti-Pre A4 monoclonal antibody (clone 22C11, raised against the extracellular domain of APP), washed 3 x 20 min, 4 x 15 min, or 5 x 5 min in TBST, and placed in sheep anti-mouse peroxidase-linked secondary antibody solution. Protein bands were visualized using a chemiluminescence method, and quantitated by laser scanning densitometry.

3.4. Immunoprecipitation and measurement of tyrosine phosphorylated proteins

Following treatment with pharmacological agents, cells were rinsed twice with phosphate-buffered saline containing 1 mM sodium orthovanadate, collected in 1 ml of lysis buffer (25 mM Tris pH 7.5, 250 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 25 μg/ml aprotinin and 2 mM 4-[2-aminoethyl]-benzenesulfonylfluoride (AEBSF)), and

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[2] APPs release was measured as described previously (Nitsch et al., 1992, and Slack et al., 1993).

placed on ice for at least 20 minutes. Lysates were centrifuged to remove the detergent-insoluble pellet and protein assays were performed to determine protein quantity in cell pellets. Tyrosine-phosphorylated proteins were precipitated from cell lysates containing 500 μg of protein by incubation with 4 μg of a polyclonal anti-phosphotyrosine antibody and 1.5 mg of protein-A Sepharose (Pharmacia Biotech Inc., Piscataway, NJ) and 1.5 mg protein-G Agarose (Oncogene Sci.) overnight at 4°C. Following centrifugation, pellets were washed 3 times in IP washing buffer (prepared identically to the lysis buffer except with 0.1% Triton X-100). Pellets were then diluted in gel loading buffer and boiled. Proteins were separated using electrophoresis and electroblotted as described previously. Membranes were incubated overnight at room temperature in a blocking buffer composed of 3% gelatin in TBST, washed 3 x 20 min in warmed up TBST, and suspended for 1 hr in a TBST solution containing a peroxidase-linked recombinant anti-phosphotyrosine antibody (Transduction Laboratories, Lexington, KY). After several washes with TBST over the course of 1 hr, bands were visualized on film using chemiluminescence.

3.5. Statistical Analysis

Values in text and figures are expressed as means ± standard error (S.E.). Statistical comparisons among multiple groups were made using one-way analysis of variance (ANOVA). Significance testing was performed using the post-hoc Tukey test, where differences were taken to be statistically significant at a p-value less than 0.05.

Chapter 4: Results

4.1. Role of Calcium Influx in APPs Secretion

Earlier experiments have demonstrated the ability of the cholinergic agonist carbachol to significantly increase the secretion of APPs (8) by binding to m1 or m3 muscarinic receptors linked to PI hydrolysis (29). Increases in the levels of IP_3 and diacylglycerol, as a result of PI breakdown, lead to the release of calcium from cellular stores and activation of PKC (10). Both PKC activation and the IP_3-triggered transient rise in intracellular calcium levels have been correlated with increased processing of APP via the secretory pathway (11, 18). However, activation of muscarinic receptors also stimulates, by a variety of mechanisms, calcium influx from the extracellular space (19, 20, 21, 22, 26), resulting in a sustained elevation in intracellular calcium. Furthermore, activation of PKC alone does not fully account for carbachol-induced APPs release, since PKC inhibition fails to completely suppress APPs secretion in cells stimulated with carbachol (fig. 2), as demonstrated previously (13). Therefore, I examined the effect of inhibiting PKC (with the bisindolylmaleimide GF109203X, a selective blocker of PKC activity) in the absence of calcium influx, achieved by chelating extracellular calcium with EGTA (fig. 2).

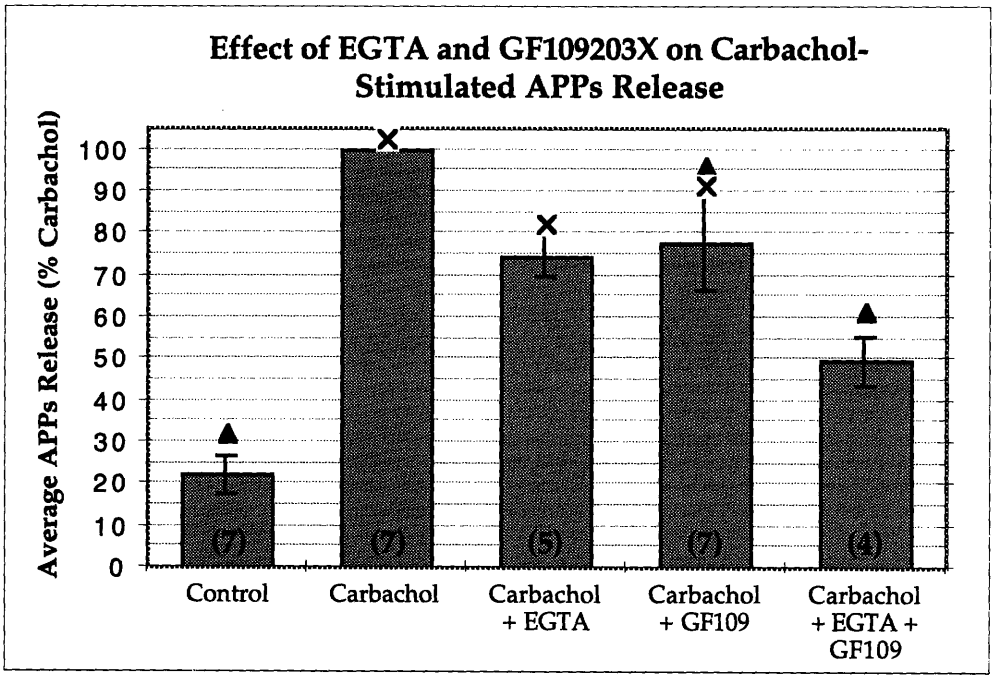
While the addition of EGTA reduced carbachol-evoked APPs release by 33%, a combination of GF109203X and EGTA caused a more significant reduction of 64%. Neither EGTA[3] nor GF109203X altered basal APPs release (13). This suggests that influx of extracellular calcium is able to modulate carbachol-induced APPs release via a mechanism independent of PKC, and

[3] APPs release during treatment with EGTA alone averaged 108 $\pm$ 4% of basal (n=4), a difference which was not significantly different from control ($p<0.05$) by paired t-test.

that these two mechanisms work in concert to mediate APPs release elicited by carbachol.

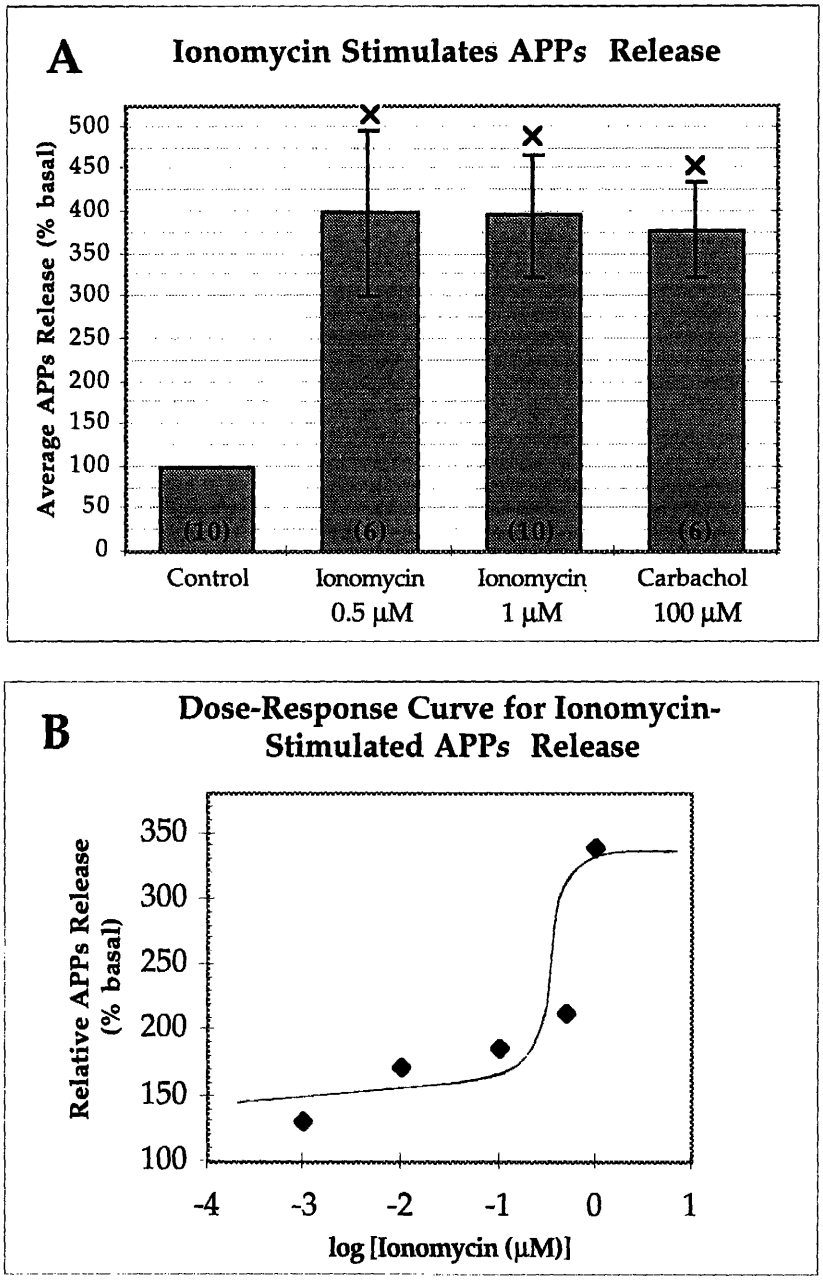
To investigate further the effect of calcium influx on APPs release, I used the ionophore ionomycin to induce calcium influx. I hypothesized that, if calcium influx accounted for part of the carbachol-mediated increase in APPs release, then directly stimulating calcium entry into the cells would

FIGURE 2



Extracellular calcium influences APPs release induced by activation of m3 receptors with carbachol. APPs released from HEK cells expressing m3 muscarinic receptors, in response to 1 hr incubation with serum-free medium or medium containing 100 μM carbachol and the indicated antagonist: 2 mM EGTA, 2.5 μM GF109203X *(GF109)*. Results are expressed as means ± S.E. from three to seven experiments performed in triplicate (n values are indicated in parentheses for each treatment group). ✖ indicates significant difference ($p<0.05$ by analysis of variance) from *Control*, ▲ indicates significant difference from *Carbachol*. The combination of a calcium chelator *(EGTA)* and a PKC inhibitor *(GF109)* reduces carbachol-evoked APPs release by over 50%, a more substantial reduction than is accomplished by either agent acting alone.

FIGURE 3



Calcium influx stimulates APPs release. **A.** APPs released during a 1 hr incubation of HEK cells transfected with m3 muscarinic receptors in serum-free control medium, medium containing 0.5 μM or 1 μM ionomycin, or 100 μM carbachol. APPs was measured by immunoblot as detailed in Section 3.2 of Experimental Procedures. Amount of APPs release is expressed as mean ± S.E. of six to ten experiments performed in triplicate (n value listed in parentheses for each treatment group). ✖ indicates significant difference from control group ($p<.05$ by analysis of variance). **B.** Ionomycin increases APPs release in a concentration-dependent manner during a 1 hr incubation. Values are averages from two experiments performed in triplicate.

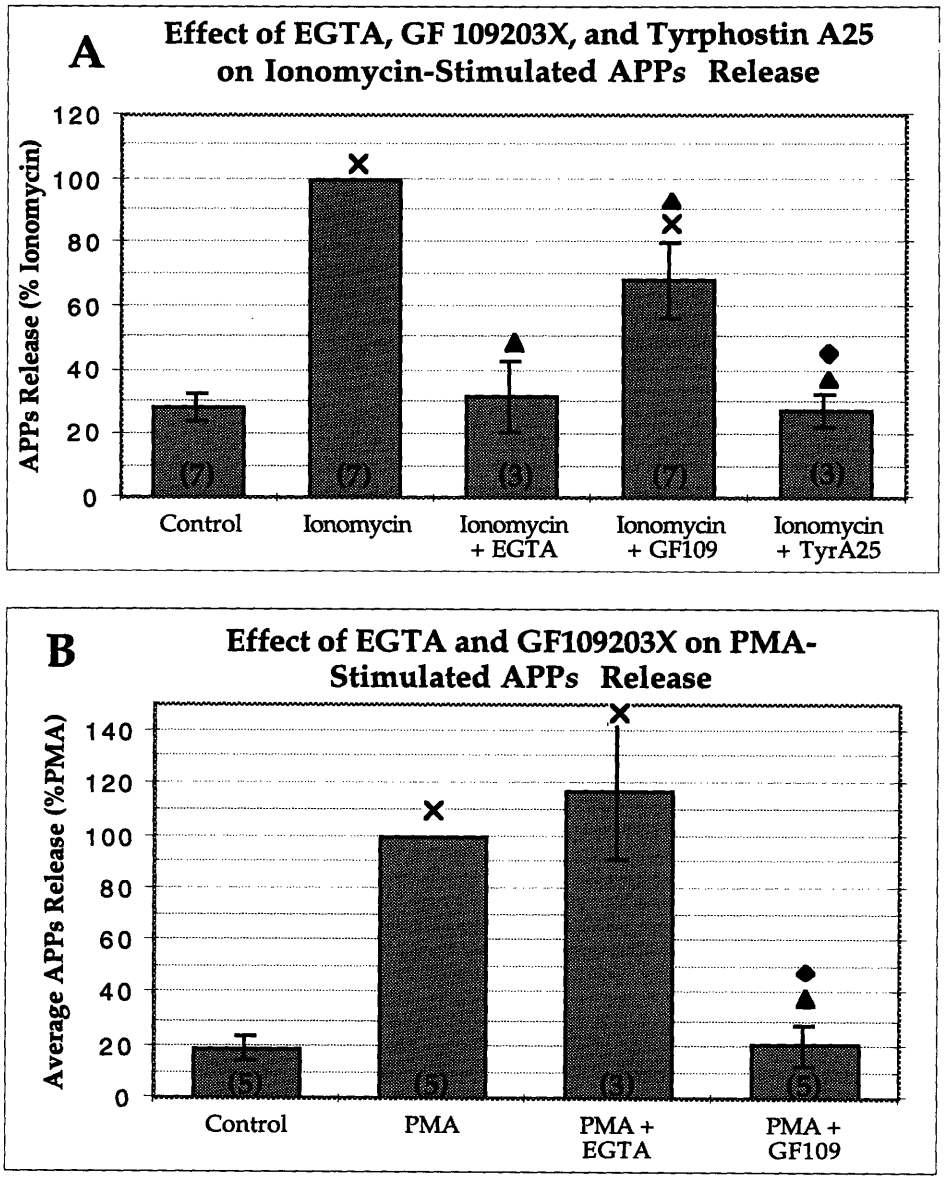
have the same effect. Ionomycin generated a 4-fold increase in APPs secretion, comparable to the effect elicited by carbachol (fig. 3a). The effect of ionomycin was also similar in magnitude to that produced by direct activation of PKC with the phorbol ester PMA (data not shown). Furthermore, ionomycin increased APPs release in a dose-dependent manner with an EC_{50} value of approximately .3 μM, and maximum secretion attained between .5 μM and 1 μM ionomycin (fig. 3b). Incubating the cells for one hour with an ionomycin concentration of 3 μM resulted in toxicity and drastically decreased the protein content as well as the release of APPs into the medium (data not shown).

To examine the involvement of PKC in the cellular signaling pathway mediating calcium influx-induced APPs secretion, I compared the effects of the PKC inhibitor GF109203X and the calcium chelator EGTA on the release of APPs elicited with ionomycin (fig 4a). The data were then contrasted with those obtained using PMA rather than ionomycin to stimulate APPs release (fig. 4b). As anticipated, EGTA and GF109203X exerted nearly reciprocal effects on ionomycin- and PMA- stimulated APPs release. Whereas EGTA blocked the effects of ionomycin, and did not affect PMA-induced APPs release, GF109203X abolished the effect of PMA, but inhibited ionomycin by only 45%. These results suggest calcium influx regulates APPs processing through both PKC-dependent and PKC-independent pathways.

In accordance with these results, I investigated the possibility that additional protein kinases mediate the PKC-independent effect of calcium influx on APPs production. It is possible that elevations in cellular calcium accompanying calcium influx activate one or more calcium/calmodulin kinases, as has been documented for IP_3-generated calcium transients (19). To test for calcium/calmodulin kinase involvement in the pathways regulating APPs secretion, I suppressed calcium/calmodulin-kinase activity

using the calmodulin inhibitor W-7 while stimulating the cells with carbachol. Under these conditions, APPs secretion did not differ markedly

FIGURE 4



Calcium influx-mediated stimulation of APPs release is contingent on tyrosine phosphorylation, and is partially dependent on PKC activation. APPs released from HEK cells expressing m3 muscarinic receptors and treated for 1 hr with serum-free medium or medium containing 1 μM ionomycin or 1 μM PMA and the indicated antagonist: 2 mM EGTA, 2.5 μM GF109203X (*GF109*), or 100 μM tyrphostin A25 (*Tyr A25*). Tyrphostin experiments required an additional pretreatment of 18-24 hrs with dimethyl sulfoxide (vehicle control) or 100 μM tyrphostin A25 in serum-free medium. Results are expressed as means ± S.E. from three to seven experiments performed in triplicate (n values are indicated in parentheses for each treatment group). ✖ indicates significant difference ($p<0.05$ by analysis of variance) from *Control*, ▲ indicates significant difference from *Ionomycin* (A) or *PMA* (B), ◆ indicates significant difference from *Ionomycin + GF109* (A) or *PMA + EGTA* (B). **A.** EGTA completely abolishes APPs release due to ionomycin, while GF109203X achieves only a partial reduction in the level of ionomycin-induced APPs release. Tyrphostin A25 completely blocks APPs release in response to ionomycin, as did EGTA, compared with only a partial block attained with GF109203X. **B.** GF109203X blocks PMA-induced APPs release, while EGTA elicits no significant effect.

from the response elicited by carbachol alone. Furthermore, application of W-7 in conjunction with GF109203X failed to limit carbachol-evoked APPs release to any greater extent than did GF109203X alone, suggesting that calcium/calmodulin-dependent kinases play little role in carbachol-mediated APPs release.

I next examined the possibility that calcium influx acts through protein tyrosine phosphorylation to mediate APPs secretion. Protein tyrosine phosphorylation has been implicated in APPs release initiated by muscarinic receptor excitation; thus, inhibition of tyrosine kinases with tyrphostin A25 significantly reduced carbachol-stimulated APPs release (13). Data from a variety of cell lines also support the hypothesis that receptor-induced tyrosine phosphorylation is mediated by receptor-associated calcium influx (20, 21, 22, 26). Consistent with this hypothesis, the specific tyrosine kinase inhibitor tyrphostin A25 consistently abolished ionomycin-stimulated APPs secretion (fig. 4a), without affecting basal APPs levels (13). The reduction achieved with tyrphostin A25 was significantly greater than the partial inhibition obtained with GF109203X. While tyrphostin A25 caused a total block of ionomycin-stimulated APPs release, it diminished PMA-evoked APPs secretion by only 37% (13).

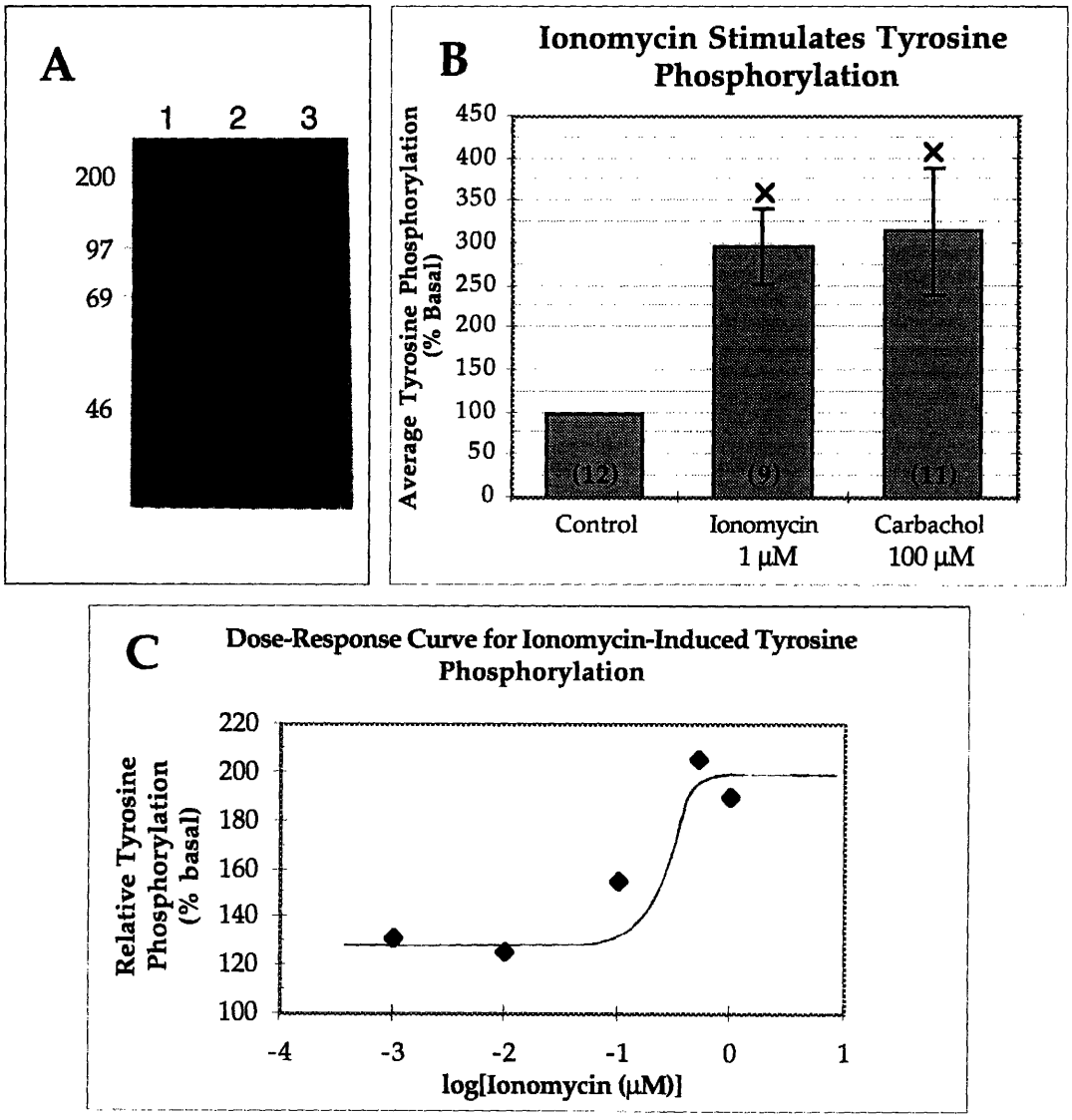
4.2. Effects of Calcium Influx on Levels of Phosphorylated Tyrosine

The effects of calcium influx on cellular levels of phosphorylated tyrosine were assessed by immunoprecipitation and western blot analysis of tyrosine-phosphorylated proteins in cell lysates. Immunoblots of tyrosine-phosphorylated proteins revealed increases in three distinct protein bands, as reported previously (13). Measurements and data analysis were performed only on the fastest migrating band representing a tyrosine-phosphorylated protein with a molecular weight of 70 kDa. Ionomycin increased phospho-tyrosine levels in a concentration-dependent manner with an EC_{50} of .2 μM, and a maximum response between .5 μM through 1 μM ionomycin; the dose-response relation for phosphorylated tyrosine paralleled that for APPs release (fig. 5b). Moreover, ionomycin stimulated tyrosine phosphorylation[4] to the same degree as did carbachol; about 3-fold with respect to basal levels (fig. 5a). In contrast, activation of PKC with PMA elicited a similar pattern of phospho-tyrosine, but to a smaller degree than was observed with ionomycin and carbachol (data not shown), consistent with earlier experiments (13).

The effects of the calcium chelator EGTA, the PKC inhibitor GF109203X, and the tyrosine kinase inhibitor tyrphostin A25 on ionomycin-evoked tyrosine phosphorylation closely correlated with their effects on ionomycin-stimulated APPs release (fig. 6). EGTA entirely blocked the increase in phosphorylated tyrosine elicited by ionomycin, as it did ionomycin-stimulated APPs release. However, GF109203X caused a more substantial suppression of the increase in tyrosine phosphorylation than of APPs secretion; 63% versus 40%. Although tyrphostin A25 abolished ionomycin-evoked APPs release, it reduced ionomycin-evoked tyrosine phosphorylation of the 70 kDa protein by 59%. None of the antagonists alone significantly attenuated basal tyrosine phosphorylation. A comparison of the effects

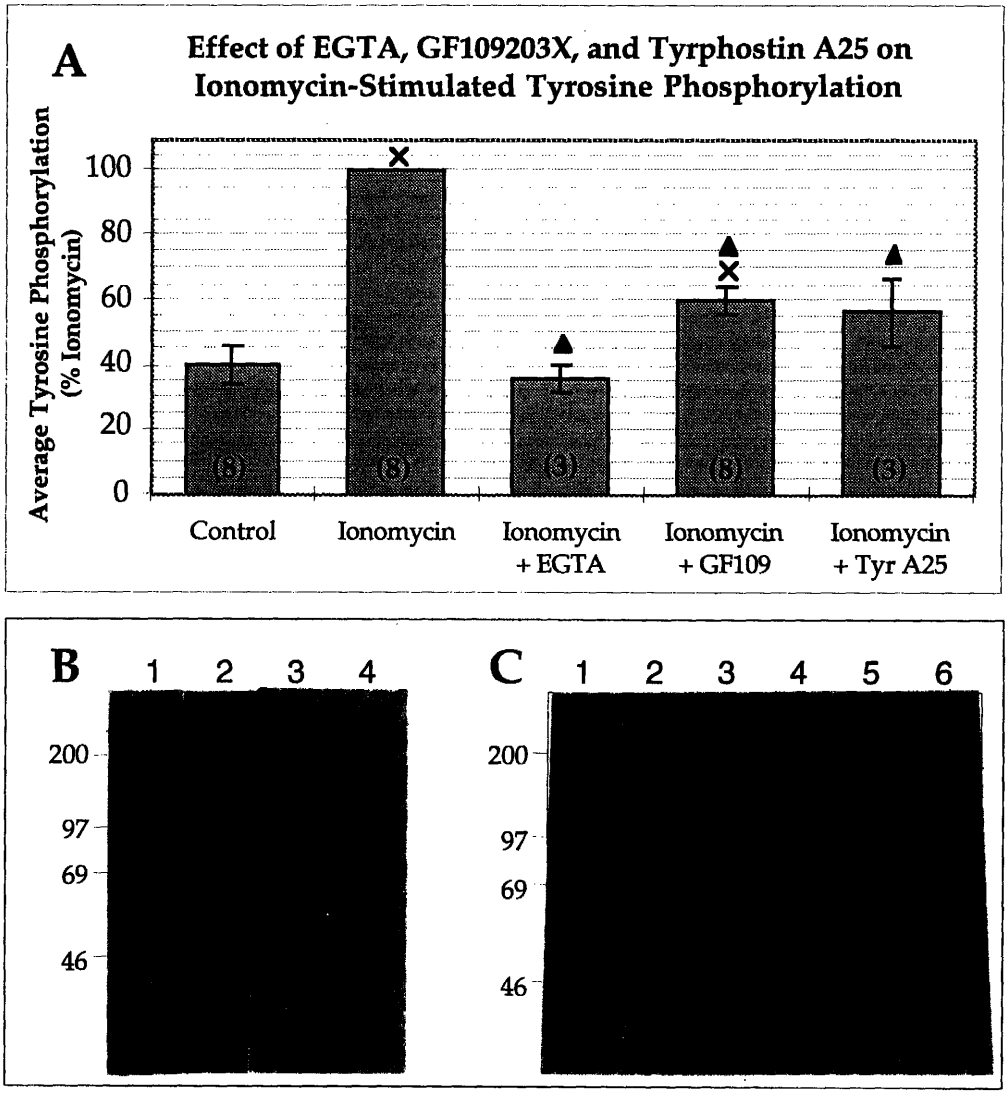
[4] In this context tyrosine phosphorylation is taken to mean levels of phosphorylated tyrosine.

FIGURE 5



Ionomycin-generated calcium influx stimulates tyrosine phosphorylation in a manner similar to carbachol. **A.** Immunoblots showing tyrosine-phosphorylated proteins in anti-phosphotyrosine immunoprecipitates from HEK cells expressing m3 muscarinic receptors. Cells were incubated for 10 min in serum-free control medium or medium containing 1 μM ionomycin or 100 μM carbachol. Ionomycin elicits a pattern of tyrosine phosphorylation similar to carbachol. Immunoblot showing three phosphotyrosine bands: 70kDa, 100kDa, 110kDa. Lanes: (1) *Control*; (2) *Carbachol*; (3) *Ionomycin*. **B.** Quantitative assessment by laser scanning densitometry of a 70 kDa molecular weight band. Phosphorylation of this band has been previously correlated to a rise in APPs release resulting from carbachol stimulation. Results are expressed as means ± S.E. from nine to twelve experiments performed in duplicate (n values are indicated in parentheses for each treatment group). ✖ indicates significant difference from *Control* ($p<0.05$ by analysis of variance). **C.** Ionomycin increases tyrosine phosphorylation of the 70 kDa band in a dose-dependent manner, as it did APPs release. Tyrosine phosphorylation of this protein appears to be slightly more sensitive to ionomycin than APPs, showing an EC_{50} value of .2 μM.

FIGURE 6

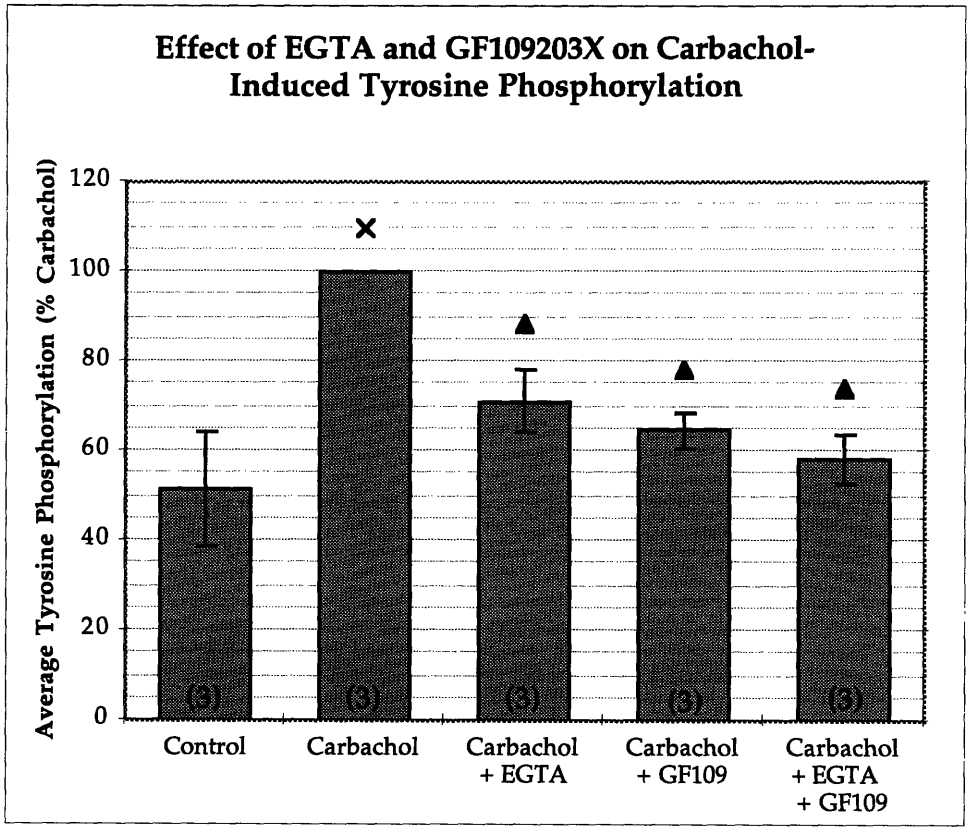


The effects of calcium chelation, PKC inhibition, and tyrosine kinase inhibition on ionomycin-stimulated tyrosine phosphorylation. Effects of 10 min incubation of HEK cells expressing m3 muscarinic receptors with serum-free medium or medium containing 1 μM ionomycin in the presence of the indicated antagonist: 2 mM EGTA, 2.5 μM GF109203X (*GF109*), 100 μM tyrphostin A25 (*Tyr A25*). Tyrphostin experiments required an additional pretreatment of 18-24 hrs with dimethyl sulfoxide (vehicle control) or 100 μM tyrphostin A25 in serum-free medium. **A.** Quantitative assessment by laser scanning densitometry of the 70 kDa molecular weight band. Results are expressed as means ± S.E. of three to eight experiments performed in duplicate (n values are listed in parentheses for each treatment group). ✖ indicates significant difference ($p<0.05$ by analysis of variance) from *Control*, ▲ indicates significant difference from *Ionomycin*. EGTA completely inhibits ionomycin-induced tyrosine phosphorylation, while GF109203X and tyrphostin A25 significantly diminish it. None of the antagonists had significant effects on basal phosphotyrosine levels. **B.** Immunoblot showing effect of inhibiting calcium influx with EGTA on tyrosine-phosphorylated proteins. Lanes: (1) *Control*; (2) *EGTA*; (3) *Ionomycin*; (4) *Ionomycin + EGTA*. **C.** Immunoblot showing effect of tyrphostin A25 and GF109203X on ionomycin-evoked increase in tyrosine-phosphorylated proteins. Lanes: (1) *Control*; (2) *Tyr A25*; (3) *GF109*; (4) *Ionomycin*; (5) *Ionomycin + TyrA25*; (6) *Ionomycin + GF109*.

of EGTA, GF109203X and tyrphostin A25 on ionomycin versus PMA-induced tyrosine phosphorylation revealed a pattern similar to the one observed with APPs release. Whereas EGTA and tyrphostin A25 generated no significant reductions in PMA-evoked tyrosine phosphorylation, GF109203X completely blocked the phosphotyrosine response to PMA (data not shown), as described previously (13). Overall, the data show that secretory processing of APP may be effectively stimulated by calcium-induced tyrosine phosphorylation.

The last set of experiments aimed to establish a correlation between carbachol-associated calcium influx, tyrosine phosphorylation, and APPs release. Carbachol has previously been shown to increase tyrosine phosphorylation concomitantly with APPs release (13), and both effects are inhibited by tyrphostin A25. Figure 7 shows that EGTA inhibits carbachol-mediated tyrosine phosphorylation, as well as APPs release. In a series of three experiments, EGTA reduced carbachol-stimulated tyrosine phosphorylation by 51%. GF109203X exhibited a more pronounced effect on carbachol-induced tyrosine phosphorylation than on APPs release, causing a 67% reduction. The combination of EGTA and GF109203X essentially abolished carbachol-stimulated tyrosine phosphorylation. The fact that these two antagonists exerted additive inhibitory effects on both APPs release and tyrosine phosphorylation, suggests that calcium influx and PKC mediate the effects of carbachol on both responses.

FIGURE 7



Removal of calcium influx with EGTA or inhibition of PKC activity with GF109203X inhibit carbachol-stimulated tyrosine phosphorylation. Results of 10 min incubation of HEK cells expressing m3 muscarinic receptors with serum-free medium or medium containing 100 μM carbachol in the presence of the listed antagonists: 2 mM EGTA, 2.5 μM GF109203X (*GF109*). Results are expressed as means ± S.E. of three experiments (n values listed in parentheses in each treatment column). ✖ indicates significant difference from control, ▲ indicates significant difference from carbachol ($p<0.05$ by analysis of variance). EGTA and GF109203X had no significant effect on basal phosphorylated tyrosine levels.

Chapter 5. Discussion

The results presented here suggest that elevations in cellular calcium levels resulting from calcium influx mediate APPs secretion stimulated by activation of muscarinic receptors linked to phosphoinositide hydrolysis. Thus, elimination of calcium influx with EGTA reduced APPs secretion by 33%. Moreover, induction of calcium influx with an ionophore stimulated APPs release in a manner that mimicked carbachol-evoked APPs secretion. Although previous work showed no response or a decline in APPs release following treatment with ionophores (8, 30), the ionophore concentrations in these studies were an order of magnitude higher than those used here. The ionomycin dose-response characteristics revealed that a concentration between 0.5 μM and 1 μM was sufficient to elicit maximal APPs release, and higher concentrations actually resulted in toxicity to the cell and consequent decline in APPs secretion. Furthermore, the calcium chelator EGTA abolished ionomycin-stimulated APPs release by preventing the flow of extracellular calcium into the cytoplasm. The manner in which muscarinic receptor activation initiates calcium influx leading to APPs secretion, whether via receptor-operated channels or IP_3-dependent depletion of intracellular calcium pools, remains to be explored.

The effects of calcium influx on APPs secretion appeared to be mediated by mechanisms which were partially independent of PKC. Consistent with this notion, the effects of EGTA and the PKC inhibitor GF109203X on APPs release were found to be additive. The combination of EGTA and GF109203X caused a 64% decrement in carbachol-stimulated APPs secretion, whereas each antagonist acting separately reduced secretion by around 30%. The ability of GF109203X to eradicate APPs release elicited by the phorbol ester PMA, a potent activator of PKC, indicates that it is a highly effective inhibitor of PKC; the specificity of GF109203X for PKC has previously been demonstrated (31). As anticipated, chelating extracellular calcium with

EGTA in no way affected PMA-induced APPs release, since PKC displays minimal requirements for calcium in the presence of this potent analog of diacylglycerol. The fact that these two antagonists exerted an additive effect on carbachol-stimulated APPs release, suggests that EGTA interfered with a PKC-independent branch of the receptor-to-APPs signaling pathway. Despite this observation, GF109203X partially blocked ionomycin-induced APPs release, suggesting that stimulation of APPs release by calcium influx proceeds in part via a PKC-dependent mechanism. This is not unexpected, since class A of PKC isozymes displays a dependence on calcium for activation. Thus, elevations in intracellular calcium of sufficient magnitude may activate some PKC subtypes at lower levels of diacylglycerol (17). However, the degree of inhibition of APPs release obtained with GF109203X was less than 50%, unlike the complete block achieved with EGTA. Therefore, calcium influx induced by ionomycin, like that elicited by carbachol, can influence APPs secretion via a signaling pathway that bypasses PKC. Finally, since the calmodulin inhibitor W-7, unlike EGTA, did not affect carbachol-stimulated APPs release, I conclude that calcium/calmodulin kinases are not involved in mediating the effects of calcium-influx on APPs release.

The experimental results further indicate that the PKC-independent signaling cascade elicits APPs release via an increase in cellular tyrosine phosphorylation levels, mediated either by activation of tyrosine kinases, or by inhibition of tyrosine phosphatases. Moreover, both the PKC-independent and -dependent pathways activated by calcium influx converge at the level of tyrosine phosphorylation, upstream of APPs release. This conclusion is based on the observation that the tyrosine kinase inhibitor tyrphostin A25 abolished ionomycin-stimulated APPs release. Tyrphostin A25 has been previously demonstrated to cause a slight reduction (37%) in PMA-induced APPs release, in contrast to the complete block attained with GF109203X (13). Therefore, the effects of calcium influx on APPs release are in part mediated by PKC activation and are contingent on subsequent tyrosine phosphorylation, yet tyrosine phosphorylation is not required for PMA to

stimulate APPs release. This apparent discrepancy may be accounted for by the potent and non-physiological activation by PMA of a wide range of PKC subtypes, not all of which may act via tyrosine phosphorylation to elicit APPs release. Calcium influx is likely to result in activation of one or more of the calcium-dependent PKC isozymes which effects APPs release via tyrosine phosphorylation. The mechanisms by which calcium influx or PKC elicits tyrosine phosphorylation were not addressed in this study.

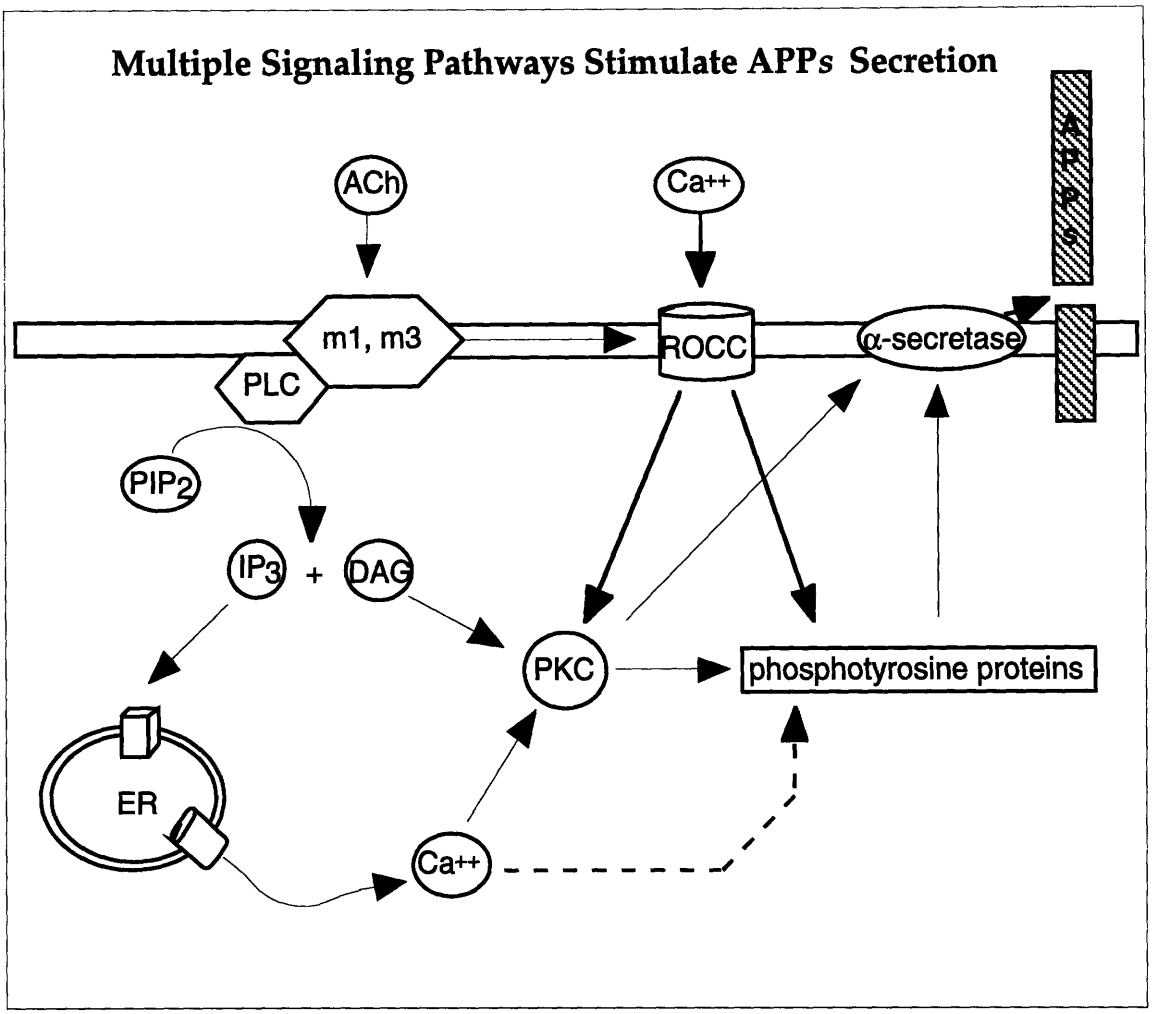
The relationship between calcium influx and tyrosine phosphorylation described above was confirmed by results obtained from anti-phosphotyrosine immunoprecipitates. Ionomycin, like carbachol, increased the phosphorylation of tyrosine residues on three proteins with molecular weights of 70 kDa, 100 kDa, and 110 kDa, discerned as separate bands on immunoblots. Moreover, the dose-response characteristics of ionomycin-induced tyrosine phosphorylation of the most prominent of these bands (70 kDa) nearly matched that obtained for APPs release. These data, in conjunction with the powerful effect of tyrphostin A25 on ionomycin-stimulated APPs release, strongly implicate calcium influx as a mediator of tyrosine phosphorylation, and subsequent APPs release.

The effects of EGTA, GF109203X, and tyrphostin A25 on ionomycin-stimulated tyrosine phosphorylation suggest that there are at least two tyrosine-phosphorylated proteins involved in mediating the effects of calcium influx on APPs release. As anticipated, chelating extracellular calcium with EGTA completely obstructed ionomycin-stimulated tyrosine phosphorylation of the 70 kDa protein, consistent with its effect on APPs release. Tyrphostin A25 had a less pronounced effect on ionomycin-induced tyrosine phosphorylation of the 70 kDa band than on APPs release, although the residual response was not significantly different from basal. However, GF109203X attenuated tyrosine phosphorylation of the 70 kDa band to a greater extent than it did APPs secretion, 60% versus 45%. The differential effect of GF109203X on APPs release and tyrosine phosphorylation of the

70 kDa protein may be explained by noting that EGTA and tyrphostin A25 effectively inhibited tyrosine phosphorylation of another protein, represented by the 100 kDa band, while GF109203X had no effect on tyrosine phosphorylation levels of this protein. These data suggests that at least two tyrosine kinases and/or phosphatases are involved in mediating the effect of calcium influx on APPs release, one of which responds to activation of one or more calcium-dependent subtypes of PKC and another which does not. Additional evidence for this conclusion has been found in previous work using the broad-spectrum protein kinase inhibitor genistein, which abolished carbachol-evoked APPs release, presumably by blocking the activity of a variety of tyrosine protein kinases (13).

A comparison of the effects of EGTA and GF109203X on carbachol-stimulated tyrosine phosphorylation versus APPs release likewise suggests that two or more tyrosine kinases and/or phosphatases with differential sensitivities to calcium-dependent subtypes of PKC are likely to mediate the effects of carbachol-generated calcium influx. Thus the effects of GF109203X on the pattern of tyrosine phosphorylation elicited by carbachol are reminiscent of its effects on ionomycin-stimulated tyrosine phosphorylation. GF109203X inhibited carbachol-stimulated tyrosine phosphorylation of the 70 kDa protein more than it did carbachol-evoked APPs release, 67% versus 33%, consistent with the differential actions of GF109203X shown in previous studies (13). However, GF109203X had little effect on tyrosine phosphorylation of the 100 kDa band induced by carbachol. These results suggest that carbachol may elicit APPs release in the absence of PKC activity by stimulating one or more PKC-independent tyrosine kinases, secondary to calcium influx. The combination of EGTA and GF109203X caused an additional, although statistically insignificant, reduction of 10% in carbachol-stimulated tyrosine phosphorylation of the 70 kDa band over that effected by GF109203X alone, indicating that phosphorylation of this band was largely PKC-mediated. The proposed mechanisms by which calcium influx influences APPs secretion are illustrated in Figure 8.

FIGURE 8



Calcium Influx and Tyrosine Phosphorylation Mediate APPs Processing. Activation of m1 or m3 muscarinic receptors by acetylcholine*(ACh)* or its analogs leads to G-protein coupled phosphoinositide *(PIP_2)* breakdown into the second messengers diacylglycerol *(DAG)* and inositol trisphosphate *(IP_3)*. IP_3 releases calcium from intracellular pools, such as the endoplasmic reticulum *(ER)*, while DAG activates protein kinase C, *(PKC)*. Class A PKC isozymes are dependent on calcium for activation. Muscarinic receptor activation also stimulates calcium entry, presumably by opening receptor-operated calcium channels *(ROCC)*. Calcium influx stimulates APPs secretion via both PKC-dependent and PKC-independent mechanisms. Whereas calcium influx regulates APPs release via phosphorylation of tyrosine on one or more proteins, PKC utilizes tyrosine phosphorylation only marginally to stimulate APPs release.

Chapter 6. Conclusions and Recommendations

This study demonstrated that calcium influx participates in the cellular signal transduction pathways that stimulate APPs release in response to the activation of muscarinic receptors. The data further reveal that APPs secretion elicited with calcium influx may be mediated by tyrosine phosphorylation independent of PKC activation, and that the PKC-dependent and independent pathways converge at the level of tyrosine phosphorylation. In a broader context, the data suggest that neurotransmitter-regulated secretory processing of APP may be relayed by multiple cellular signaling cascades, which recruit common messenger proteins. Thus, calcium influx and PKC signaling cascades postulated here do not necessarily serve as exclusive mediators of muscarinic receptor activation on APPs release. Evidence for an additional pathway was suggested by the significant elevation in carbachol-mediated APPs release that persisted in the presence of EGTA and GF109203X. However, the remaining response was further reduced by tyrphostin A25, suggesting that it is the result of residual, possibly calcium-independent, tyrosine phosphorylation-dependent activity.

Although this study postulated a role for calcium influx in APPs processing, it is possible that elevations in cellular calcium brought about by other mechanisms will also induce APPs release. For instance, other studies (18) showed that increases in cellular calcium levels mediated by IP_3 may also stimulate APPs secretion independent of PKC. It is therefore possible that the source contributing to calcium elevations, whether intracellular or extracellular pools, may be of little relevance to the final response. However, it is also possible that APPs processing has a preferential requirement for one calcium source over another. Moreover, these sources of calcium may act together to reinforce the release of APPs, since calcium release from internal stores produces a fast-acting transient while calcium influx results in a slower

rising, but sustained elevation. Whether the IP_3-generated calcium transients stimulate tyrosine phosphorylation remains to be investigated.

The next step in elucidating the muscarinic receptor-to-APPs signaling pathway to identify whether increase in phospho-tyrosine levels are the result of activation of tyrosine kinases, inhibition of tyrosine phosphatases, or both. The subsequent task will be to identify the enzymes and substrates that participate in regulating APPs release. Finally, the mechanism by which these signaling molecules trigger secretory cleavage of APPs release remain to be elaborated. One hypothetical mechanism might involve phosphorylation of the cytoplasmic domain of the APPs molecule by a protein kinase, rendering it susceptible to cleavage by a transmembrane secretase. However, significant evidence indicates otherwise (32). Another possible mechanism of APP metabolism involves phosphorylation and consequent activation of the α-secretase responsible for cleavage of APP into APPs and other derivatives. A third mechanism of APP cleavage may not rely on sudden activation of α–secretase but instead harness its basal activity. Tyrosine kinases exhibit a regulatory domain which allows them to attach to each other to form active regulatory complexes. Stimulation of tyrosine phosphorylation by muscarinic receptor activation may induce the assembly of such a multi-kinase complex at a site on the plasma membrane. This regulatory complex might serve as a docking mechanism for α-secretase (provided it possesses a regulatory domain), bringing it into proximity with APP and enabling cleavage to take place.

These findings have significant implications for the mechanisms by which neuronal cells protect themselves against injury. When a cell experiences increases in calcium influx due to excitotoxic insults, it may respond by stimulating secretory processing of APP. APP could then act as a neuroprotective agent, stabilizing intracellular calcium concentrations. In Alzheimer's disease, the postulated shift in APP processing in favor of Aβ may prevent neurons from properly responding to the neurotoxic effects of

aggregated Aβ. The consequent disruption of cellular calcium homeostasis would then precipitate cell death.

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