Elevated intracellular calcium concentration increases secretory processing of the amyloid precursor protein by a tyrosine phosphorylation-dependent mechanism

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Secretory cleavage of the amyloid precursor protein (APP), a process that releases soluble APP derivatives (APPs) into the extracellular space, is stimulated by the activation of muscarinic receptors coupled to phosphoinositide hydrolysis. The signalling pathways involved in the release process exhibit both protein kinase C- and protein tyrosine phosphorylation-dependent components [Slack, Breu, Petryniak, Srivastava and Wurtman (1995) J. Biol. Chem. **270**, 8337–8344]. The possibility that elevations in intracellular Ca^{2+} concentration initiate the tyrosine phosphorylation-dependent release of APPs was examined in human embryonic kidney cells expressing muscarinic m3 receptors. Inhibition of protein kinase C with the bisindolylmaleimide GF 109203X decreased the carbachol-evoked release of APPs by approx. 30%, as shown previously. The residual response was further decreased, in an additive manner, by the Ca^{2+} chelator EGTA, or by the tyrosine kinase inhibitor tyrphostin A25. The Ca^{2+} ionophore, ionomycin, like carbachol, stimulated both the release of APPs and the tyrosine phosphorylation of several proteins, one of which was identified as paxillin, a component of focal adhesions. The effects of ionomycin on APPs release and on protein tyrosine phosphorylation were concentration-dependent, and occurred over similar concentration ranges; both effects were inhibited only partly by GF 109203X, but were abolished by EGTA or by tyrosine kinase inhibitors. The results demonstrate for the first time that ionophore-induced elevations in intracellular Ca^{2+} levels elicit APPs release via increased tyrosine phosphorylation. Part of the increase in APPs release evoked by muscarinic receptor activation might be attributable to a similar mechanism.

INTRODUCTION

One of the hallmarks of the pathology of Alzheimer's disease is the presence of senile plaques in the brains of patients with the disease. These plaques are composed of aggregated peptides, (amyloid beta peptide; 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. This secretory processing event leads to the release of a large soluble fragment of APP (APPs) into the extracellular space and precludes the formation of Aβ [2–4]. Alternatively, APP can be cleaved in intracellular compartments, forming potentially amyloidogenic C-terminal fragments [5–7]. A third metabolic pathway gives rise to soluble Aβ peptides [8–10], which may aggregate into amyloid plaques under certain, incompletely defined conditions. Because APPs and Aβ seem to be formed by two mutually exclusive mechanisms [11–13], stimulation of secretory processing of APP might prevent the formation of Aβ and its accumulation into amyloid plaques. Although other evidence indicates that in some cell types the release of APPs and Aβ can proceed independently of each other [13,14], the ability of APPs to protect neurons against excitotoxic and ischaemic insults [15,16] provides an additional basis for the hypothesis that the sustained release of APPs is important for the maintenance of neuronal viability.

The release of APPs is increased by the activation of muscarinic cell-surface receptors linked to phosphoinositide (PI) hydrolysis [17–19]. Stimulation of m1 and m3 muscarinic receptor subtypes activates PI-specific phospholipase C, which in turn catalyses the breakdown of PI to diacylglycerol and inositol 1,4,5-trisphosphate. The formation of diacylglycerol at the cytosolic face of the plasma membrane, in conjunction with the release of Ca^{2+} from internal stores elicited by inositol trisphosphate, activates protein kinase C (PKC) [20]. Direct activation of PKC by phorbol esters, which mimic the effects of diacylglycerol, has been shown to release APPs [21–23], raising the possibility that this enzyme mediates the effects of muscarinic receptor stimulation on APP processing. However, inhibition of PKC with the specific PKC inhibitor GF 109203X only partly suppressed the APPs release induced by a muscarinic agonist, carbachol [24], and the release of APPs stimulated by the muscarinic agonist bethanechol was unaffected by prior down-regulation of PKC [25]. Both GF 109203X and chronic exposure to phorbol esters specifically block phorbol ester-responsive forms of PKC [26,27], implicating additional signalling mechanisms in this process. We proposed a role for tyrosine phosphorylation, based on evidence that carbachol-induced elevations in tyrosine phosphorylation were correlated with increases in APPs release; that carbachol's effects were decreased by tyrosine kinase inhibitors; and that increases in tyrosine phosphorylation induced by tyrosine phosphatase inhibitors stimulated APPs release [24].

Evidence that muscarinic receptor activation increases tyrosine phosphorylation via Ca^{2+} influx [28,29] suggested that elevations in intracellular Ca^{2+} concentration might initiate tyrosine phosphorylation-dependent APPs release in response to carbachol. This hypothesis was tested in human embryonic kidney (HEK)

Abbreviations used: Aβ, amyloid beta peptide; APP, amyloid precursor protein; APPs, soluble APP; HEK, human embryonic kidney; PI, phosphoinositide; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TBST, Tris-buffered saline with 0.15% (v/v) Tween-20.

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Table 1 Additive inhibition of carbachol-induced release of APPs by GF 109203X and EGTA, or by GF 109203X and tyrphostin A25

HEK cells expressing m3 muscarinic receptors were incubated for 1 h with serum-free control medium or medium containing 100 μM carbachol in the presence or absence of 2.5 μM GF 109203X, 5 mM EGTA or 100 μM tyrphostin A25 (TA25; see Experimental section). APPs was measured by immunoblot as detailed in the Experimental section. Results are means $\pm$ S.E.M. for the numbers of experiments indicated in parentheses, performed in triplicate. *Significant difference from carbachol; †significant difference from carbachol + EGTA; ‡significant difference from carbachol + TA25 (by ANOVA followed by Fisher's least significant difference test). Abbreviation: n.d., not done.

	APPs release (% of carbachol response)	
Treatment	Control	GF 109203X (2.5 μM)
Control	17.5 $\pm$ 3.1 (13)*	n.d.
Carbachol	100 (13)	67.9 $\pm$ 8.1 (11)*
Carbachol + EGTA	74.6 $\pm$ 4.8 (5)*	49.8 $\pm$ 5.8 (4)*†
Carbachol + TA25	68.6 $\pm$ 4.8 (6)*	38.8 $\pm$ 5.2 (6)*‡

cells expressing muscarinic m3 receptors. The results showed that the activation of muscarinic receptors by carbachol increased APPs release in part by a mechanism that involved increased tyrosine phosphorylation secondary to Ca^{2+} entry, and was independent of phorbol ester-sensitive PKC isoforms. Moreover, APPs release elicited by the Ca^{2+} ionophore ionomycin was entirely dependent on tyrosine phosphorylation.

EXPERIMENTAL

Materials

Phorbol 12-myristate 13-acetate (PMA), EGTA and carbachol were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.); tyrphostin A25, tyrphostin A1, genistein and GF 109203X (bisindolylmaleimide) were purchased from LC Laboratories (Woburn, MA, U.S.A.), and ionomycin was purchased from Calbiochem (La Jolla, CA, U.S.A.). Stock solutions were prepared by dissolving compounds in DMSO, then further diluting in serum-free medium. When Ca^{2+} chelation was required, EGTA was added to serum-free medium. A concentration of 5 mM was sufficient to abolish the effects of ionomycin. For measurement of APPs release, cells were incubated in test solutions for 30 or 60 min. For phosphotyrosine determinations, cells were treated for 10 min. Cells were preincubated with tyrphostin A25, an inactive analogue tyrphostin A1, or an equivalent concentration of the vehicle (DMSO) for 18–24 h before the administration of acute treatments as described below. Cells were pretreated with genistein for 10 min. Final DMSO concentrations did not exceed 0.2% (v/v).

Cell culture

HEK 293 cell lines stably transfected with m3 muscarinic receptors (a gift from Dr. Ernest Peralta) were used as a model to study the effects of Ca^{2+} on APPs release. HEK cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 bicarbonate medium supplemented with 10% (v/v) fetal calf serum and grown in an atmosphere of 5% CO_2 in air. Test incubations were conducted in serum-free Dulbecco's modified Eagle's medium buffered with bicarbonate [24].

Measurement of APPs release

Measurement of APPs release was performed as detailed previously [17,24]. After incubation with test solutions, media were centrifuged to remove debris and treated with the protease inhibitor PMSF. Media were desalted, freeze-dried and suspended in SDS/PAGE sample buffer. Cells were harvested in PBS, centrifuged, then lysed in a buffer containing 1% (v/v) Triton X-100 [24]. Lysates were centrifuged to remove detergent-insoluble material and diluted 1:1 in sample buffer. Media and lysate samples were boiled, then separated on 12% (w/v) polyacrylamide mini-gels (Bio-Rad, Richmond, CA, U.S.A.). Proteins were electroblotted on poly(vinylidene difluoride) membranes, which were then blocked with 5% (w/v) powdered milk in Tris-buffered saline with 0.15% (v/v) Tween-20 (TBST), and immunoblotted with anti-(Pre A4) monoclonal antibodies (clone 22C11; Boehringer Mannheim, Indianapolis, IN, U.S.A.) or monoclonal antibody 6E10 (Senetek, Maryland Heights, MO, U.S.A.). After being washed, membranes were incubated with a peroxidase-linked secondary antibody (Amersham, Arlington Heights, IL, U.S.A.). Bands were revealed by a chemiluminescence method, and quantified by laser scanning densitometry (LKB). In some experiments, a Molecular Dynamics densitometer and ImageQuant software were used for quantification. The same pattern of agonist-evoked APPs release was observed whether 22C11 antibodies, which recognize both APP and APP-like proteins [30], or 6E10 antibodies, which are specific for APP [31], were used (Figure 1A).

Immunoprecipitation and measurement of tyrosine-phosphorylated proteins

Immunoprecipitation of tyrosine-phosphorylated proteins was performed as described previously [24]. After treatment with pharmacological agents, cells were rinsed twice with PBS containing 1 mM sodium orthovanadate, then collected in 1 ml of lysis buffer [25 mM Tris (pH 7.5)/250 mM NaCl/5 mM EDTA/1% (v/v) Triton X-100/1 mM sodium orthovanadate/25 μg/ml aprotinin/2 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride]. Lysates were centrifuged and tyrosine-phosphorylated proteins were precipitated from cell lysates containing 500 μg of protein by incubation with 4 μg of polyclonal anti-phosphotyrosine antibodies (Upstate Biotechnology, Lake Placid, NY, U.S.A.) or 4 μg of monoclonal anti-phosphotyrosine antibodies (clone PY20, Transduction Laboratories, Lexington, KY, U.S.A.), with 1.5 mg of Protein A–Sepharose (Pharmacia Biotech, Piscataway, NJ, U.S.A.) and 1.5 mg of Protein G–agarose (Oncogene Science, Cambridge, MA, U.S.A.) overnight at 4 °C. In some experiments, immunoprecipitates were prepared by using monoclonal antibodies to paxillin (Transduction Laboratories; 4 μg per sample). Immunoprecipitates were washed three times in a washing buffer [prepared in the same way as the lysis buffer but with 0.1% (v/v) Triton X-100], diluted in sample buffer and boiled. Proteins were separated by electrophoresis and electroblotted as described above. Membranes were blocked with 3% (w/v) gelatin in TBST and probed with peroxidase-linked recombinant anti-phosphotyrosine antibodies (Transduction Laboratories), or with anti-paxillin antibodies followed by peroxidase-linked anti-mouse secondary antibodies. After several washes with TBST, bands were detected on film by a chemiluminescence method. Prestained molecular mass standards (Amersham) were routinely used to monitor protein transfer, but molecular masses of tyrosine-phosphorylated proteins were estimated with biotinylated standards (Bio-Rad, Hercules, CA, U.S.A.).

Statistical analysis

Values in the text and in figures are expressed as means $\pm$ S.E.M.

Statistical comparisons between groups were made by one-way analysis of variance (ANOVA). Significance testing was performed with Fisher's least significant difference test; differences were taken to be statistically significant at $P < 0.05$.

RESULTS

Carbachol-mediated APPs release is dependent on PKC activation, tyrosine phosphorylation and Ca^{2+} influx

It was shown previously that the cholinergic agonist carbachol significantly increased the secretion of APPs [17] by activating m1 or m3 muscarinic receptors linked to PI hydrolysis [32,33]. This effect of carbachol was partly decreased (by approx. 40 %) by GF 109203X (Table 1) [24], an inhibitor of phorbol ester-sensitive isoforms of PKC [26], and decreased further by treatment with the Ca^{2+} chelator EGTA and GF 109203X together (Table 1). The same concentration of GF 109203X (2.5 μM) abolished PMA-induced APPs release (Table 2). Carbachol-stimulated APPs release was also additively inhibited by a combination of GF 109203X and the tyrosine kinase inhibitor tyrphostin A25 (Table 1). None of GF 109203X, tyrphostin A25 [24] or EGTA significantly altered the basal release of APPs. [APPs release during treatment with EGTA alone averaged 108 ± 4 % of basal ($n = 4$), a difference that was not significantly different from the control ($P < 0.05$) by paired t test.] These results indicate that elevations in intracellular Ca^{2+} concentration and tyrosine phosphorylation might influence the carbachol-induced release of APPs independently of phorbol ester-responsive isoforms of PKC.

Table 2 Ionomycin-evoked release of APPs is dependent on tyrosine phosphorylation and PKC activation

HEK cells expressing m3 muscarinic receptors were treated for 30 or 60 min with serum-free control medium or medium containing ionomycin or PMA, with or without the indicated compounds: 5 mM EGTA, 2.5 μM GF 109203X, 100 μM tyrphostin A25 (TA25) or 100 μM tyrphostin A1 (TA1; see Experimental section). Results are means ± S.E.M. for the numbers of experiments indicated in parentheses. *Significant difference from control; †significant difference from ionomycin or PMA alone; ‡significant difference from ionomycin + GF 109203X (by ANOVA followed by Fisher's least significant difference test). Abbreviation: n.d., not done.

		APPs release (% of maximal response)	
Treatment	Activator ...	Ionomycin (1 μM)	PMA (1 μM)
Control		28.2 ± 3.0 (10)†	18.8 ± 4.4 (5)†
Activator		100 (10)*	100 (5)*
Activator + EGTA		32.0 ± 11.2 (3)†‡	117 ± 25.6 (3)*
Activator + GF 109203X		68.1 ± 12.5 (7)*†	20.7 ± 7.5 (5)†
Activator + TA25		36.6 ± 5.3 (6)†‡	n.d.
Activator + TA1		81.1 ± 8.2 (3)*	n.d.

Ionomycin stimulates APPs release

To test the possibility that Ca^{2+} influx and tyrosine phosphorylation are sequential steps in APP release, the ionophore ionomycin was used to increase intracellular Ca^{2+} concentrations, and APPs release and tyrosine phosphorylation were assessed. Ionomycin elicited a 3-fold increase in APPs release, an effect comparable to that of carbachol and PMA (Figure 1A). The results were almost identical whether immunoblotting was performed with 22C11 (Figure 1A, upper panel) or 6E10 (Figure 1A, lower panel) monoclonal antibodies; the latter recognize amino acid residues 1–16 of the Aβ domain of APP and therefore are specific for APP [31]. Western blots made with 22C11 were used for quantitative analysis. APPs release evoked by ionomycin was concentration-dependent and approached maximal levels at ionomycin concentrations between 0.5 and 1 μM (Figure 1B).

Ionomycin-induced APPs release is blocked by EGTA and tyrosine kinase inhibitors

Ionomycin- and PMA-stimulated APPs releases were distinct in terms of their sensitivities to the inhibitors EGTA and GF 109203X (Table 2). Whereas EGTA abolished the effect of

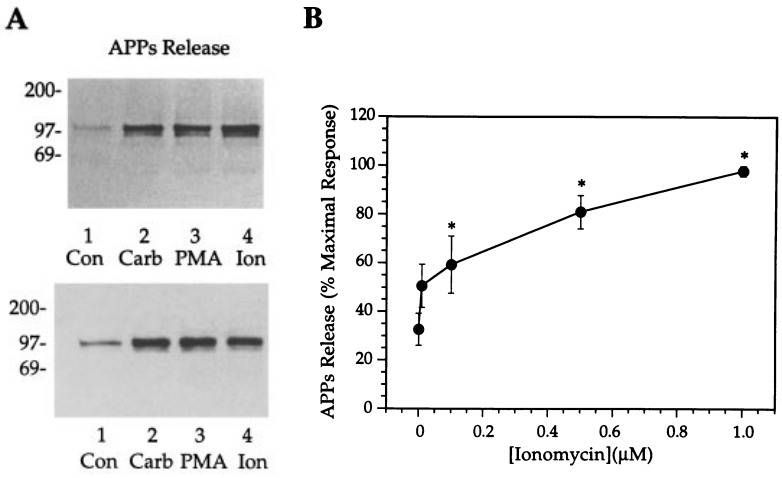

Figure 1 Ionomycin stimulates APPs release

(**A**) Western blot analysis of APPs released from HEK cells expressing m3 muscarinic receptors, incubated for 1 h in serum-free medium or in medium containing 100 μM carbachol (Carb), 1 μM PMA or 1 μM ionomycin (Ion). Immunoblots were made with 22C11 (upper panel) or 6E10 (lower panel) monoclonal antibodies. (**B**) APPs released from cells treated for 1 h with varying concentrations of ionomycin. Values are expressed as means ± S.E. for five to nine experiments. *Significant difference from control by ANOVA followed by Fisher's least significant difference test.

Table 3 Ionomycin-induced APPs release is inhibited by genistein

Cells were pretreated for 10 min with 50 μM genistein or vehicle (DMSO) then incubated for 30 min in control medium or in medium containing 0.5 μM ionomycin in the presence or absence of 50 μM genistein. Results are means $\pm$ S.D. for replicate determinations from 2 experiments. *Significant difference from control; †significant difference from ionomycin (by ANOVA followed by Fisher's least significant difference test).

Treatment	APPs release (% of ionomycin response)
Control	56.2 $\pm$ 20.4†
Ionomycin	100*
Genistein	37.1 $\pm$ 9.1*†
Ionomycin + genistein	38.8 $\pm$ 4.7*†

ionomycin and had no effect on the PMA-induced release of APPs, GF 109203X blocked the effect of PMA but inhibited ionomycin's effect by less than 50%. The tyrosine kinase inhibitors tyrphostin A25 (Table 2) and genistein (Table 3) abolished ionomycin-stimulated APPs secretion. In contrast with its effect on ionomycin, tyrphostin A25 decreased the PMA- and carbachol-evoked increase in APPs secretion by approx. 40% (Table 1) [24]. The inactive analogue tyrphostin A1 did not significantly inhibit APPs release (Table 2).

Ionomycin increases tyrosine phosphorylation

These results suggested that ionomycin induced APPs release by increasing tyrosine phosphorylation; its ability to stimulate tyrosine phosphorylation was therefore measured directly. Previous results showed that carbachol increased the phosphotyrosine content of two prominent protein bands with estimated molecular masses of 70 and 112 kDa [24]. Ionomycin elicited an identical response (Figure 2A). On 7.5% (w/v) gels, the larger band was resolved into two bands with approximate molecular masses of 102 and 112 kDa (Figure 2B, upper panel). The 70 kDa protein was identified as paxillin on the basis of the following evidence: (1) a protein of the same size was immunoprecipitated by antibodies to paxillin and co-migrated on SDS gels with the 70 kDa protein immunoprecipitated by anti-phosphotyrosine antibodies (Figure 2B, upper panel); (2) the protein immunoprecipitated by antibodies to paxillin showed increased tyrosine phosphorylation in response to treatment with ionomycin and carbachol (Figure 2B, upper panel, lanes 5–8); (3) anti-phosphotyrosine immunoprecipitates from ionomycin- and carbachol-treated cells, but not from controls, contained a 70 kDa protein that was detected by antibodies to paxillin (Figure 2B, lower panel); and (4) the 70 kDa tyrosine-phosphorylated protein was not present in anti-phosphotyrosine immunoprecipitates made with lysates that had been precleared with anti-paxillin antibodies (results not shown). Ionomycin increased the phosphotyrosine content of the paxillin-containing band (measured in anti-phosphotyrosine immunoprecipitates) in a concentration-dependent manner, with a maximum response at 0.5 μM ionomycin (Figure 2C); the dose–response relationship for phosphorylated tyrosine was very similar to that for APPs release. PMA elicited a similar pattern of tyrosine phosphorylation, but the effect was smaller than that observed with ionomycin and carbachol (results not shown), consistent with earlier experiments [24].

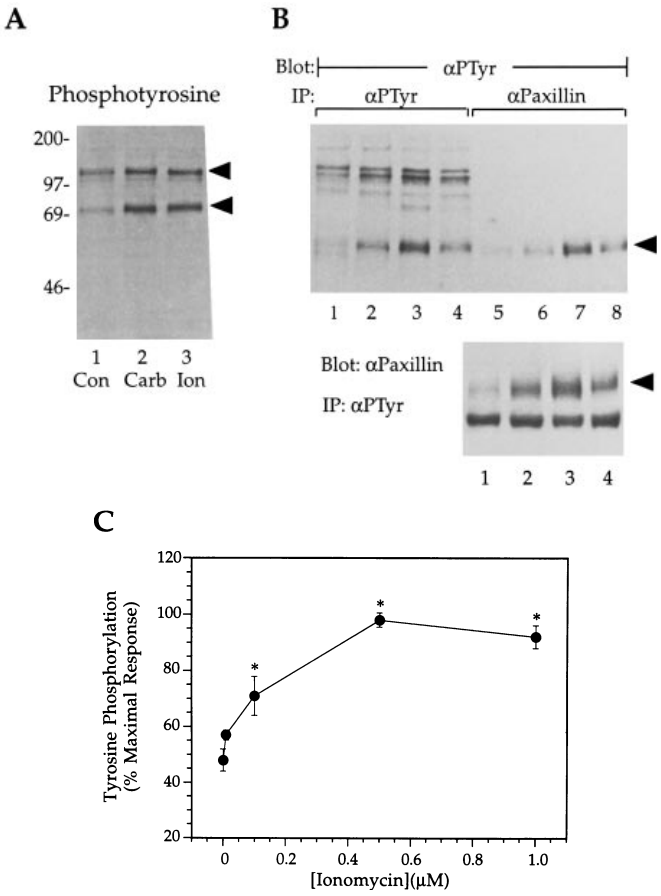

Figure 2 Ionomycin increases tyrosine phosphorylation

(**A**) Cells were incubated for 10 min in serum-free control medium (Con) or in medium containing 100 μM carbachol (Carb) or 1 μM ionomycin (Ion). Tyrosine-phosphorylated proteins were immunoprecipitated from cell lysates with anti-phosphotyrosine antibodies, size-fractionated on 12% (w/v) gels and immunoblotted with anti-phosphotyrosine antibodies. The two most prominent bands (arrowheads) have approximate molecular masses of 70 and 112 kDa. (**B**) Upper panel: cells were treated for 10 min with control medium (lanes 1 and 5) or medium containing 0.5 μM ionomycin (lanes 2 and 6), 100 μM carbachol (lanes 3 and 7) or 1 μM ionomycin (lanes 4 and 8). Proteins immunoprecipitated with antibodies to phosphotyrosine (lanes 1–4) or to paxillin (lanes 5–8) were size-fractionated on 7.5% (w/v) gels and immunoblotted with antibodies to phosphotyrosine. Lower panel: cells were treated as in the upper panel (lanes 1–4). Tyrosine-phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibodies and immunoblotted with antibodies to paxillin. Arrowheads indicate the position of paxillin. The band below paxillin represents the heavy chain of the immunoprecipitating antibody. (**C**) Ionomycin increased tyrosine phosphorylation of the 70 kDa band in a concentration-dependent manner. Values are means $\pm$ S.E.M. for three or four experiments. *Significant difference from control, by ANOVA followed by Fisher's least significant difference test.

Ionomycin-induced tyrosine phosphorylation is decreased by GF 109203X, EGTA and inhibitors of tyrosine kinase in parallel with APPs release

If tyrosine phosphorylation and APPs release are causally related sequential events, they should be affected in a similar manner by antagonists acting upstream of the phosphorylation event. EGTA completely blocked both the increase in tyrosine phosphorylation and APPs release induced by ionomycin (Tables 2 and 4). GF 109203X decreased the ionomycin-evoked increase in tyrosine phosphorylation of the 70 kDa/paxillin band by 66%, and inhibited the increase in APPs release by 44% (Tables 2 and 4). Tyrphostin A25 decreased the ionomycin-evoked increase

Table 4 Effects of inhibitors on ionomycin- and carbachol-evoked tyrosine phosphorylation

The table shows phosphorylation of the 70 kDa band (paxillin) after 10 min incubation of HEK cells expressing m3 muscarinic receptors with serum-free control medium or medium containing 1 μM ionomycin or 100 μM carbachol in the presence of the indicated compounds: 5 mM EGTA, 2.5 μM GF 109203X or 100 μM tyrphostin A25 (TA25; see Experimental section). Results are means $\pm$ S.E.M. for the number of experiments indicated in parentheses, performed in duplicate. None of the inhibitors had a significant effect on basal phosphotyrosine levels. *Significant difference from control; †significant difference from ionomycin or carbachol (by ANOVA followed by Fisher's least significant difference test). Abbreviation: n.d., not done.

		Tyrosine phosphorylation (% of maximal response)	
Treatment	Activator ...	Ionomycin	Carbachol
Control		40.2 $\pm$ 6.0 (8)†	51.3 $\pm$ 12.7 (3)†
Activator		100 (8)*	100 (3)*
Activator + EGTA		35.7 $\pm$ 4.4 (3)†	71.2 $\pm$ 7.2 (3)†
Activator + GF 109203X		60.4 $\pm$ 4.3 (8)*†	64.7 $\pm$ 3.7 (3)†
Activator + GF 109 + EGTA		n.d.	58.2 $\pm$ 5.6 (3)†
Activator + TA25		56.7 $\pm$ 10.7 (3)†	n.d.

in APPs release by 90% (Table 2) and inhibited the increase in tyrosine phosphorylation of the 70 kDa paxillin-containing band by 72% (Table 4). The tyrosine kinase inhibitor genistein (50 μM) blocked the ionomycin-induced release of APPs (Table 3) and decreased the ionomycin-evoked increase in tyrosine phosphorylation of the 70 kDa band by 59 $\pm$ 8% ($n = 3$; results not shown). These inhibitors exerted similar effects on the phosphorylation by ionomycin of the 112 and 102 kDa bands, with the exception that the evoked increase in phosphorylation of the 102 kDa band was resistant to GF 109203X (results not shown).

Carbachol-induced tyrosine phosphorylation is inhibited by EGTA and GF 109203X

EGTA and GF 109203X, which decreased the carbachol-mediated release of APPs (Table 1), also caused a significant inhibition of carbachol-mediated tyrosine phosphorylation (Table 4). The two inhibitors affected APPs release in a nearly additive fashion; although a similar trend was observed with respect to the inhibition of tyrosine phosphorylation, the effect of the two inhibitors combined was not significantly greater than the effect of either alone. Tyrphostin A25 caused a partial inhibition of both carbachol-mediated APPs release and tyrosine phosphorylation [24].

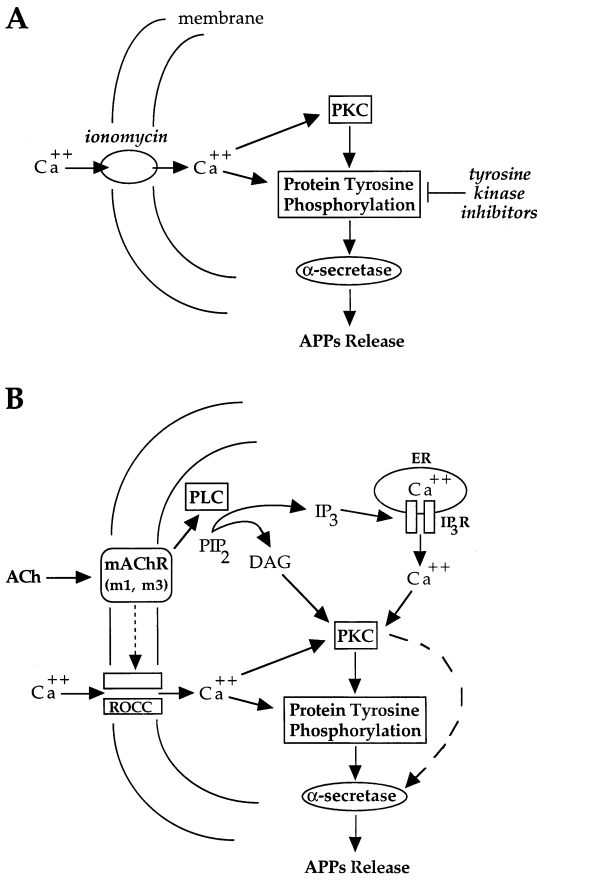

Figure 3 Postulated signalling pathways regulating APPs release evoked by ionomycin and carbachol

(**A**) APPs release evoked by ionomycin is partly dependent on PKC activity (i.e. it is partly inhibited by GF 109203X), but is abolished by the tyrosine kinase inhibitors tyrphostin A25 or genistein, suggesting that several alternative pathways might converge at a tyrosine phosphorylation-dependent step. (**B**) This model is derived from results described here and in previous publications (see the text). Activation of m1 or m3 muscarinic receptors by acetylcholine (ACh) or its analogues leads to G-protein-coupled phosphoinositide (PIP_2) breakdown to form the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP_3). IP_3 acts on its receptor (IP_3R) to release Ca^{2+} from the endoplasmic reticulum (ER), whereas DAG activates PKC. Muscarinic receptor activation might also stimulate Ca^{2+} entry, possibly via receptor-operated Ca^{2+} channels (ROCC). Ca^{2+} influx increases APPs secretion via both PKC-dependent and tyrosine phosphorylation-dependent mechanisms. Because PMA-induced APPs release is only partly reduced by tyrphostin A25, the possibility remains that additional (possibly Ca^{2+}-independent) PKC isoforms regulate APPs release by an alternative mechanism (broken arrow). The possibility that one or more phorbol ester-insensitive isoforms of PKC are components in the tyrosine phosphorylation-dependent pathway has not been ruled out.

DISCUSSION

These results suggest that elevations in intracellular Ca^{2+} concentration and increased tyrosine phosphorylation represent sequential steps in the stimulation of APPs release by ionomycin (Figure 3A), and account for part of the response to the muscarinic agonist carbachol (Figure 3B). It was previously shown that activation of muscarinic receptors coupled to phosphoinositide hydrolysis stimulated APPs release by both PKC- and tyrosine phosphorylation-dependent mechanisms [24]. The results described here indicate that the latter pathway is initiated by elevations in intracellular Ca^{2+} concentration; this is possibly secondary to increased Ca^{2+} influx, and does not depend on the activation of phorbol ester-sensitive isoforms of PKC, given that both EGTA and the tyrosine kinase inhibitor tyrphostin A25 caused decrements in the carbachol-mediated release of APPs that were additive to those caused by GF 109203X. (These results do not rule out the possibility that phorbol ester-insensitive isoforms of PKC, e.g. PKCζ, also contribute to the carbachol response.) Increasing intracellular Ca^{2+} concentration with ionomycin stimulated both APPs secretion and tyrosine phosphorylation. These effects of ionomycin were inhibited by the tyrosine kinase inhibitors tyrphostin A25 and genistein. Thus activation of the undefined protease α-secretase might be contingent on tyrosine phosphorylation, possibly of α-secretase itself, or of an intermediate protein in the signalling pathway. It is unlikely that the direct phosphorylation of APP is part of this process, because deletion of the intracellular portion of the molecule does not inhibit evoked secretory processing [34,35].

Ca^{2+}-dependent increases in tyrosine phosphorylation after the activation of muscarinic receptors have been previously described in Chinese hamster ovary cells transfected with m5 muscarinic receptors [28]. Moreover, others have shown, with the same line of m3 receptor-expressing HEK cells used in the present study, that the stimulation of phospholipase D activity by carbachol could be partly reduced by PKC inhibitors but it was virtually abolished by the tyrosine kinase inhibitor genistein [36]. Thus a variety of processes initiated in these cells by muscarinic receptor activation are dependent on both PKC and tyrosine phosphorylation.

Although in an earlier study the ionophore A23187 did not affect APPs release [17], the concentration tested (10 μM) was an order of magnitude higher than in the present study, in which ionomycin concentrations of 0.5 and 1 μM were sufficient to elicit a 3-fold increase in APPs release. A higher concentration (3 μM) resulted in significant cell detachment and was not tested further. Others have shown that A23187 at a concentration of 0.5 μM significantly increased Aβ release from HEK 293 cells stably transfected with APP [37] but did not affect the production of APPs. This might have been due to interference with the production of mature APP by A23187, as indicated by the appearance of an isoform of intermediate molecular mass [37], although basal release of the corresponding APPs intermediate was not affected.

In contrast with A23187, the tumour promotor thapsigargin, a compound that increases cytoplasmic Ca^{2+} levels by inhibiting Ca^{2+} re-uptake into intracellular stores, stimulated the release of APPs [25]. The response was unchanged after down-regulation of PKC by prolonged exposure to phorbol 12,13-dibutyrate. (This treatment, like that with GF 109203X, would be expected to block only phorbol ester-sensitive forms of the enzyme.) Thapsigargin has been shown to increase protein tyrosine phosphorylation in platelets [38] and to induce transmembrane Ca^{2+} influx in a variety of cell types [39–41]. The rise in Ca^{2+} concentration induced by thapsigargin is decreased by tyrosine kinase inhibitors [39–41]. Taken together, the results demonstrate that elevations in intracellular Ca^{2+} effectively stimulate APPs release and raise the possibility that thapsigargin, like ionomycin, stimulates APPs release via a tyrosine phosphorylation-dependent mechanism.

Chelation of extracellular Ca^{2+} with EGTA blocked the APPs release induced by ionomycin and decreased the effect of carbachol, yet had no affect on PMA-induced release (Table 2); this finding was not unexpected, because PKC displays minimal requirements for Ca^{2+} in the presence of this potent analogue of diacylglycerol. On the other hand, GF 109203X partly decreased the ionomycin-induced release of APPs, indicating that at least under certain conditions the stimulation of APPs release by Ca^{2+} influx can proceed in part via the activation of PKC. This suggests the involvement of Group A (conventional) PKC isozymes [20], which depend on Ca^{2+} for activation; indeed, PKCα has been implicated in the regulation of APPs release in fibroblasts [23]. Elevations in intracellular Ca^{2+} concentration of sufficient magnitude might activate these PKC subtypes at lower levels of diacylglycerol than are typically required [42].

Although GF 109203X only partly inhibited the ionomycin-evoked release of APPs, tyrphostin A25 abolished it, indicating that both PKC-dependent and PKC-independent components of the response, as pictured in Figure 3A, converge on an obligatory tyrosine phosphorylation-dependent step. Because the response to PMA is largely (approx. 60%) resistant to tyrphostin A25 [24], it is likely that ionomycin stimulates a subset of PKC isoforms that increase APPs release by acting on tyrosine kinases or phosphatases that are intermediates in the regulatory pathway. Alternatively, ionomycin might recruit PKC isoforms that are activated by tyrosine phosphorylation. However, although carbachol and PMA promote tyrosine phosphorylation of PKCδ in salivary gland epithelial cells [43], the effects of phosphorylation on enzyme activity remain ambiguous. Moreover, ionomycin did not phosphorylate PKCδ [43], suggesting that our results might best be explained by postulating the existence of a tyrosine phosphorylation-dependent step downstream of PKC (Figure 3A).

Ionomycin, like carbachol, significantly increased the phosphorylation on tyrosine residues of two major proteins with molecular masses of 70 and 112 kDa and a minor one of 102 kDa, discerned as separate bands on immunoblots. The 70 kDa band has been identified as paxillin, a 68 kDa protein involved in the formation of focal adhesions. The latter are complexes of cytoskeletal-associated proteins recruited to the cytoplasmic face of attachment sites formed by the binding of integrins to the extracellular matrix [44]. Cytoskeletal rearrangement and increased tyrosine phosphorylation of paxillin and other components of focal adhesions are induced by cell attachment, by growth factors and by the activation of receptors belonging to the G-protein-coupled, seven-transmembrane-domain receptor superfamily [45–47]. In the present study the phosphorylation state of paxillin was increased in parallel with APPs release, raising the possibility that physiological stimulation of APPs release might occur in conjunction with the cytoskeletal reorganization that accompanies cell attachment. This hypothesis is supported by the observation of Beyreuther and co-workers that APPs release is increased in adherent microglial cells, relative to that in non-adherent or poorly adherent cells [48], and is consistent with the proposed role of APPs as an adhesion molecule [49,50]. Although the identities of the other tyrosine-phosphorylated proteins described in this study are not yet known, our results strongly suggest that one or more of them might participate in the secretory processing of APP. It also remains to be determined which tyrosine kinases or phosphatases mediate the observed increases in tyrosine phosphorylation. Indirect evidence for the existence of Ca^{2+}-activated tyrosine kinases [29,38,51], recently reinforced by the cloning of PYK2, a Ca^{2+}-dependent tyrosine kinase found mainly in brain [52], points to members of this family as potential regulators of APP processing.

In summary, the results show that neurotransmitter-regulated secretory processing of APP is relayed by multiple signalling cascades, and that an intermediate step in the stimulation of APPs release by agents that elevate intracellular Ca^{2+} concentration involves the tyrosine phosphorylation of one or more proteins.

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