Expression of the endocytic proteins dynamin and amphiphysin in rat gastric enterochromaffin-like cells

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Summary

Dynamin and amphiphysin play crucial roles in a variety of endocytic processes. Previous investigations of expression and functions of these proteins were performed mostly on neurons. The aim of this study was to investigate the presence and interaction of dyn and amph in gastric enterochromaffin-like cells. These endocrine cells of the gastric mucosa play a pivotal role in the regulation of acid secretion. Exocytosis of histamine-containing secretory vesicles has been described in detail. However, the mechanisms of endocytosis are unknown in this neuroendocrine cell type. Using RT-PCR and western blotting, we detected dynamin-1, -2 and -3 in highly enriched isolated enterochromaffin-like cells. Dynamin-1 and -2 were expressed at similar high levels, whereas dynamin-3 was of low abundance. Immunofluorescence microscopy located dynamin-1 and -2 to the cytoplasm and cell surface, whereas dynamin-3 was distributed differently in the perinuclear area. The presence of amphiphysin-1 and -2 RNAs was revealed by RT-PCR and a new splice variant of amphiphysin-2 was detected. Amphiphysin-1 and -2 were also detected in enterochromaffin-like cells by immunohistochemistry in the same locations as dynamin-1 and -2. Amphiphysin-1 and dynamin-1 co-immunoprecipitated with amphiphysin-2. In addition, dynamin-1 and amphiphysin-2 partially colocalized at the plasma membrane. Our results confirm the interaction of dynamin and amphiphysin and imply a role in endocytosis in enterochromaffin-like cells. To our knowledge, this is the first demonstration of the co-expression of all three dynamin isoforms in a non-tumor cell.

Key words: Amphiphysin, Dynamin, ECL cells, Endocytosis

Introduction

Dynamins and amphiphysins are multimodular proteins that are crucial for endocytic processes (Marsh and McMahon, 1999; McPherson, 1999; Slepnev and De Camilli, 2000). Dynamins are 100 kDa GTPases that mediate membrane fission (McNiven et al., 2000a; Schmid et al., 1998; Hinshaw, 2000). The exact mechanism of dynamin action is still a matter of debate. Data support the role of dynamin either as a mechanoenzyme or a regulator for downstream effectors (Sever, 2002; Thompson and McNiven, 2001). In mammals, three dynamin (dyn) isoforms have been detected so far. These isoforms show extensive alternative splicing and are distributed in a tissue-specific manner. Dyn1 is found in neuronal cells (Obar et al., 1990), dyn2 is expressed ubiquitiously (Cook et al., 1994; Sontag et al., 1994) and dyn3 is restriced to testis, brain, heart and lung (Nakata et al., 1993; Cao et al., 1998; Cook et al., 1996).

Amphiphysins are thought to direct dynamins to sites of endocytosis by interacting with dynamin, clathrin and clathrin adaptor proteins (Wigge and McMahon, 1998; Takei et al., 1999). In mammals, the expression products of two amphiphysin (amph) genes are present. Amph1 is found in brain and adrenal glands (Lichte et al., 1992) and in lower amounts in Sertoli cells of the testis (Ramjaun et al., 1997; Watanabe et al., 2001). The extensively spliced amph2 is expressed ubiquitously, with specific expression of some splice forms in neurons, muscle cells and macrophages (Wigge et al., 1997; Ramjaun et al., 1997; Butler et al., 1997; Gold et al., 2000).

Most of the insights into the function of dynamin and amphiphysin have come from studies using neuronal cell models. However, there is little knowledge about the expression of these proteins in gastric cells. Only the presence of dynamin in parietal cells has been reported previously, where it has been found to be localized to the apical membrane (Calhoun et al., 1998; Okamoto et al., 2000). In the current study, we investigated the presence and interaction of dynamin and amphiphysin in gastric enterochromaffin-like (ECL) cells. These neuroendocrine cells are located in the basal third of the gastric corpus glands and play an important role in the peripheral regulation of mammalian gastric acid secretion (Prinz et al., 1999). On stimulation with the antral hormone gastrin, ECL cells secrete histamine, which is stored in numerous secretory vesicles with a characteristic electron-dense core (Fig. 1) (Prinz et al., 1993; Prinz et al., 1994). Histamine acts as a paracrine stimulus of acid secretion by binding to H_2 receptors present on gastric parietal cells. Exocytosis of histamine from ECL cells is a Ca^{2+}-regulated process which is mediated by the SNARE proteins synaptobrevin and SNAP-25 (Zanner et al., 2002; Höhne-Zell et al., 1997).

In the current study, we found that in gastric ECL cells, all dynamin and amphiphysin isoforms are expressed at the

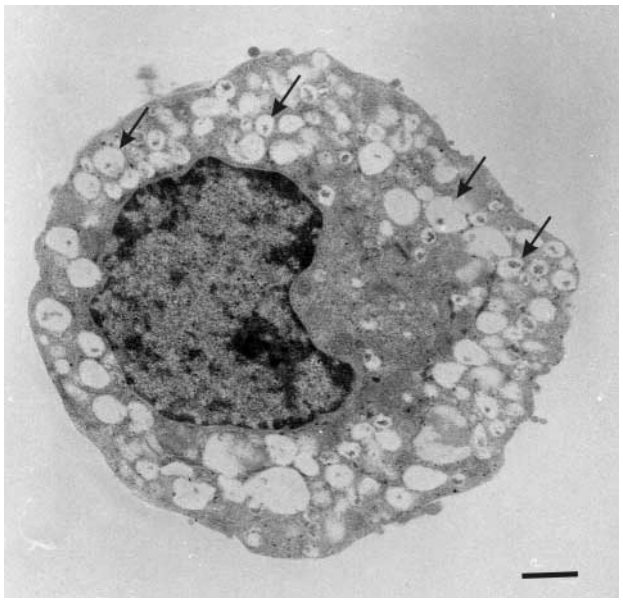


Fig. 1. Typical electron micrograph of an isolated ECL cell. Numerous secretory vesicles are present, some of them containing an electron-dense core (arrows). Bar, 1 μm.

mRNA and protein levels. We also discovered a novel splice variant of amphiphysin-2. Furthermore, we observed similar subcellular distributions of dynamins and amphiphysins in ECL cells and interaction of amphiphysins and dyn1 by co-immunoprecipitation. These data underline the importance of these endocytotic proteins in ECL cells and thereby in the regulation of acid secretion.

Materials and Methods

Reagents and antibodies

Pronase and protein-A agarose were from Roche Molecular Biochemicals (Mannheim, Germany), Nycodenz from Accurate Chemicals (Westbury, NJ), fetal bovine serum and ultrapure agarose from Gibco BRL Life Technologies GmbH (Karlsruhe, Germany) and bovine serum albumine from Serva (Heidelberg, Germany). DME-Ham's F-12 medium, dithiothreitol, acridine orange, trypan blue, gentamicin, hydrocortisone, rat gastrin-17, insulin, transferrin, sodium selenite and Tween 20 were purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). Cell-Tak was from Becton Dickinson (Heidelberg, Germany) and Triton X-100 from BioRad (Munich, Germany). All chemicals were of analytical grade.

Monoclonal antibodies directed against dynamin-1 were obtained from Transduction Laboratories (Lexington, KY) and Upstate Biochtechnology (Lake Placid, NY). Mouse monoclonal antibodies against amphiphysin-1 were purchased from Transduction Laboratories and Stressgen (San Diego, CA). Rabbit polyclonal antibodies against dynamin-2 (anti-Dyn2T) and dynamin-3 (anti-Dyn3T) were kindly provided by Mark A. McNiven (Mayo Clinic, Rochester, MN). The polyclonal antibody against amphiphysin-2 (1874) was a kind gift of Peter S. McPherson (McGill University, Montreal, Canada) and the polyclonal antibody against histidine decarboxylase was purchased from Eurodiagnostica (Malmö, Sweden). Secondary horseradish peroxidase (HRP)-labeled IgG antibodies were from Amersham Pharmacia Biotech Europe GmbH (Freiburg, Germany). Secondary IgG antibodies linked to Alexa Fluor™ 488 and Alexa Fluor™ 647 were purchased from Molecular Probes (Eugene, OR).

Isolation and culture of ECL cells

ECL cells were isolated from rat stomach as previously described (Prinz et al., 1994; Höhne-Zell et al., 1997; Zanner et al., 2002). For each experiment, five female Sprague Dawley rats (body weight 180-200 g; Charles River, Sulzfeld, Germany) were anaesthetized by carbon dioxide and killed by cervical dislocation in accordance with the ethics guidelines of the Technische Universität München, Germany. The stomachs were excised and prepared by the everted sac technique. Enzymatically dispersed cells (pronase E, 1.3 mg/ml) were fractionated by counterflow elutriation (JE-6B elutriation rotor run in a J2-21M/E centrifuge, Beckman Instruments, Munich, Germany) and subsequently subjected to Nycodenz density gradient centrifugation. Enriched ECL cells were cultured for 48 hours at 37°C as indicated in DME/F-12 supplemented with 5% fetal bovine serum, 2 mg/ml sterile bovine serum albumine, 5 mg/l gentamicin, 1 nM hydrocortisone, 5 mg/l insulin, 5 mg/l transferrin, 5 μg/l sodium selenite and 1 pM gastrin, pH adjusted to 7.38. This cultured cell preparation was made up of more than 90% ECL cells, as determined by acridine orange uptake and specific antibody staining using the polyclonal antibody against the marker enzyme histidine decarboxylase, as reported previously (Prinz et al., 1993; Prinz et al., 1994; Höhne-Zell et al., 1997; Mahr et al., 1998).

Isolation of RNA, reverse transcription and PCR

Total RNA was isolated from enriched ECL cells using peqGOLDTriFast reagent (Peqlab Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's instructions. Complementary DNA was generated from total RNA with Taq Man Reverse Transcription Reagents from Perkin Elmer (Weiterstadt, Germany). The coding sequences for dynamin and amphiphysin isoforms published in GenBank™ were used to generate subtype specific oligonucleotide primers (Table 1).

PCR for dynamin isoforms and amph1 was performed using Qiagen Master Mix Kit (Qiagen, Hilden, Germany) and the following temperature cycle profile: 1 minute at 94°C, 30 cycles for 45 seconds at 94°C, 55 seconds at specific primer annealing temperature (55°C for dyn1, 60°C for dyn2, 58°C for dyn3 and amph1), and 1 minute at 72°C. The profile was ended with a final extension of 10 minutes at 72°C.

Table 1. Oligonucleotide primer sequences

Gene	Accession number	Forward primer	Reverse primer	Product (bp)
Dyn1	X54531	5′-GAC CAG ATC GAC ACT TAT GAA-3′	5′-GCC ACC CCT TTC GAA TG-3′	533
Dyn2	L24562	5′-ATC AAG TCG ACA CCC TGG AG-3′	5′-ACC AGC CCC TGC GGA TC-3′	531
Dyn3	D14076	5′-CCA CCA CCC AAA GAA GGC T-3′	5′-CTG GAT GGA ACC TGT GGA G-3′	190
Amph1	Y13381	5′-CTG GAG GTG AAG AAG GAG G-3′	5′-TCA GTT CCC ACA GTC TCA CC-3′	398
Amph2	Y13380	5′-GGA TGG CAG AGA TGG GGA GCA AGG G-3′	5′-ATG GAA GTT TTC CTC CAG ACC CGC GAT GC-3′	*
Amph2 full	Y13380	5′-GGA TGG CAG AGA TGG GGA GCA AGG G-3′	5′-TCA CAC ACC GGA AGG CTG CAG AGG C-3′	*

*These primer pairs produced several bands due to alternative splicing.

Amphiphysin-2 was amplified using the cDNA GC Polymerase Mix (Clontech, Palo Alto, CA) and the following temperature cycle profile: 1 minute at 94°C, 35 cycles for 30 seconds at 94°C, and 3 minutes at 66°C. The profile was ended with a final extension of 10 minutes at 72°C.

Amplified products were subjected to horizontal agarose gel electrophoresis and documented with PhotoFinish PC software (WordStar Atlanta Technology Center) using a video documentation system (MWG Biotech, Ebersberg, Germany). Representative bands were eluted from the gel, cloned into TOPO pCR 2.1 vector (Invitrogen, Groningen, Netherlands) and sequenced for verification.

Protein extraction

Isolated and purified ECL cells were washed in cold PBS and subjected to centrifugation for 5 minutes at 1000 r.p.m. Cells were lysed in 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1% Triton X-100 supplemented with protease inhibitor cocktail (Calbiochem, La Jolla, CA) for 30 minutes and sonicated briefly. Cellular debris was removed by microcentrifugation. All steps were carried out at 4°C. Proteins from PC12 cells, chief cells and rat brain/liver, which served as controls, were extracted in the same way. The protein concentration of each sample was determined with Coomassie brilliant blue using a BioRad protein assay (BioRad Laboratories, Munich, Germany).

Immunoprecipitation

Total protein extracts were incubated in 30 μl of protein-A agarose slurry for 2 hours on a rocking platform to remove unspecific binding. The supernatant was then incubated for 3 hours with 2 μg of amph2 (1874) antibody prebound to protein-A agarose. Immunoprecipitates were washed several times in lysis buffer and eluted in Laemmli buffer. All steps were carried out at 4°C. Negative controls were performed using unrelated IgG antibodies and did not produce any bands.

SDS-PAGE and western blot analysis

Total protein samples or immunoprecipitates were separated by continuous SDS-PAGE on 7.5% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (BioRad Laboratories, Munich, Germany). After blotting, filters were blocked in TBS-Tween containing 5% low fat milk and incubated with primary antibodies diluted in blocking buffer. Primary antibody binding was detected using anti-mouse or anti-rabbit secondary antibodies conjugated to HRP (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) diluted in blocking buffer. Reactive bands were visualized using the enhanced chemiluminescence detection method (Amersham Pharmacia).

Immunohistochemistry of rat gastric mucosa

A rat stomach was fixed by immersion in 4% (wt/vol) paraformaldehyde in PBS for 2 days at 4°C, subsequently washed for 12 hours in PBS, supplemented with 6.8% (wt/vol) sucrose and embedded in paraffin. Tissue sections were deparaffinized in xylene, rehydrated in an ethanol series and heated in a microwave oven twice in 10 mM citrate, pH 6.0. Endogenous peroxidase activity was blocked with 10% methanol and 0.3% H_2O_2 in PBS for 10 minutes. Subsequently, the sections were incubated in blocking buffer (5% normal goat serum in PBS), avidin and biotin for 30 minutes each and afterwards in primary antibody solution overnight at 4°C. After rinsing in PBS, tissue sections were covered with biotinylated secondary antibody solution for 1 hour at room temperature and once again washed in PBS. For signal detection, avidin-labeled peroxidase (Vectastain, Vector Laboratories, Burlingame, CA) and diaminobenzidine were used.

Indirect immunofluorescence microscopy

Isolated ECL cells were cultured on sterilized glass coverslips precoated with Cell-Tak diluted 1:1 with 0.5 M $NaHCO_3$ and cultured for 2 days. Fixation was performed in 4% paraformaldehyde at room temperature for 10 minutes. After blocking with 10% normal serum, the cells were incubated with primary antibody solution over night at 4°C. After washing, cells were incubated with anti-mouse or anti-rabbit secondary antibodies conjugated to Alexa Fluor™ 488 for 1 hour at room temperature and processed for confocal microscopy.

For double labeling immunocytochemistry, ECL cells were incubated with solution containing monoclonal anti-dyn1 and polyclonal anti-amph2 antibodies overnight at 4°C. After washing, cells were incubated with anti-mouse antibodies conjugated to Alexa Fluor™ 647 for 1 hour at room temperature and after an additional washing step with anti-rabbit antibodies conjugated to Alexa Fluor™ 488. For better contrast, the blue colour of Alexa Fluor™ 647 was changed to red using the LSM 5 Imaging software (Carl Zeiss, Göttingen).

Electron microscopy

Isolated ECL cells were cultured in Petri dishes precoated with Cell-Tak diluted 1:1 with 0.5 M $NaHCO_3$ and cultured for 2 days. After brief rinsing in PBS, cells were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer, pH 7.4 overnight, postfixed in 1% OsO_4, embedded in Epon and processed for electron microscopy. Sections were examined with the electron microscope EM10 (Carl Zeiss, Göttingen).

Results

Expression of dynamin in isolated ECL cells

mRNAs of the three known dynamin isoforms were amplified from highly enriched ECL preparations using isoform-specific oligonucleotide primers (Table 1). As shown in Fig. 2, we found RNAs for dyn1, dyn2 and dyn3 in ECL cells. Dyn3 seems to be the least expressed isoform in ECL cells, since PCR for this isoform produced the weakest band when compared with the positive control (brain/PC 12 cells). These data are in accordance with our investigation of protein expression of dynamin isoforms in isolated ECL cells by western blotting. As shown in Fig. 3, all three isoforms were detected. Dyn1 and dyn2 both produced strong signals after short exposure times. Dyn3 expression could only be detected using immunoprecipitation. Thus, our immunoblotting experiments confirm our results obtained by RT-PCR.

Immunolocalization of dynamin isoforms in isolated ECL cells

To investigate the intracellular distribution of dynamin isoforms, we performed immunoflourescence staining on isolated ECL cells using isoform-specific antibodies. Cells were observed in a confocal microscope. The monoclonal Hudy-1 antibody directed against dyn1 produced an immunostaining of the cytoplasm and bright spots at the plasma membrane (Fig. 4A). Dyn2 immunoreactivity was also found in the cytoplasm, close to the nucleus and the plasma membrane (Fig. 4B). Dyn3 was mainly detected in the perinuclear area (Fig. 4C). All antibodies used for

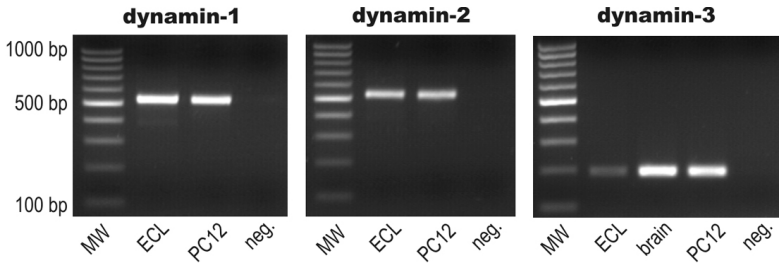


Fig. 2. RT-PCR for dynamin isoforms. All three isoforms were present in ECL cells. Positive (PC12 cells/brain) and negative controls are also shown. MW, molecular weight markers.

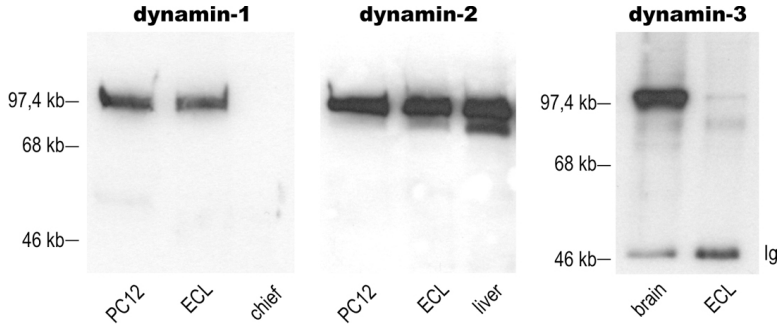


Fig. 3. Western blot analysis of dynamin protein expression in ECL cells. Dyn1 and dyn2 produced strong signals after short exposure times, whereas dyn3 was barely detectable. Exocrine chief cells served as a negative control for the dyn1 antibody. Ig, immunoglobulins.

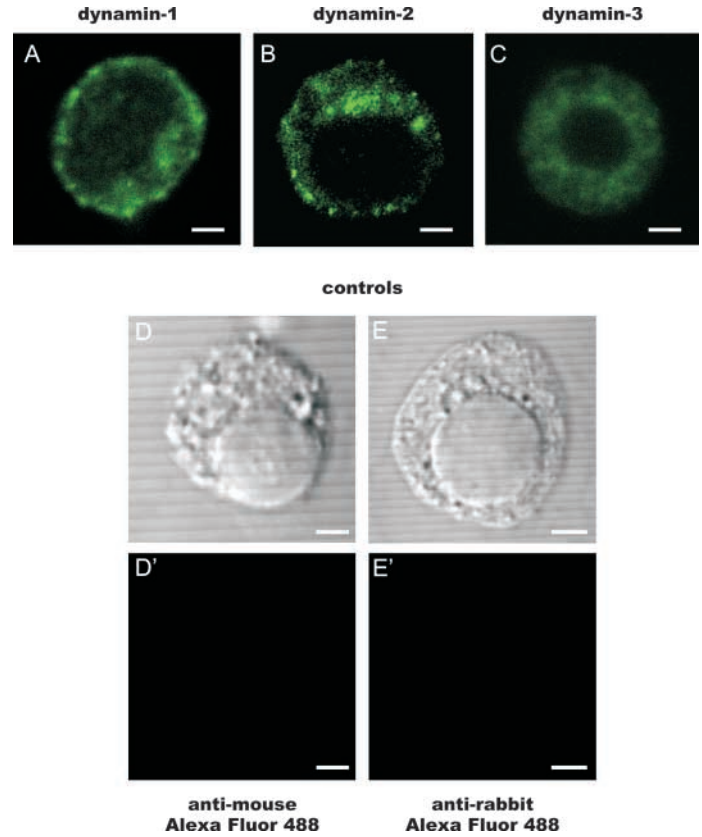


Fig. 4. Immunolocalization of dynamin isoforms in isolated ECL cells using subtype-specific antibodies. Dyn1 immunoreactivity was found in the cytoplasm and at the plasma membrane (A). Dyn2 immunoreactivity was also found in the cytoplasm, the perinuclear region and more strongly at the plasma membrane (B). Dyn3 was mainly detected in the perinuclear area (C). Negative controls (secondary antibodies only) did not produce unspecific staining (D-E'). Bars, 2 μm.

immunofluorescence experiments are specific and have been used in a variety of studies (Damke et al., 1994; Warnock et al., 1995; Cao et al., 1998). Negative controls (secondary antibodies only) are depicted in Fig. 4D-E'.

Immunolocalization of amphiphysin in gastric ECL cells

Immunohistochemistry on sections of paraffin-embedded tissue from the acid producing part of the rat stomach was used to identify cells expressing amphiphysin isoforms. Fig. 5A-C shows low power magnification of sections stained with a monoclonal antibody against amph1 (Fig. 5A), a polyclonal antibody against amph2 (Fig. 5B) and a polyclonal antibody against histidine decarboxylase (HDC), the marker enzyme for ECL cells (Fig. 5C). With the amph1 antibody, weak staining of few cells at the base of the gastric glands could be observed, as well as stronger staining of neurons of the enteric nervous system, which were also positive for the neuronal markers AP180 and syntaxin (data not shown).

The staining pattern observed with the amph2 antibody basically resembled that of the antibody against amph1. With the polyclonal antibody against HDC, a strong and specific staining of mucosal ECL cells could be observed (Fig. 5C).

To identify exactly the amph1- and amph2-positive cells in the gastric mucosa, we perfomed immunohistochemistry of consecutive thin sections with these antibodies and the antibody against HDC. As shown in Fig. 5D,D' and Fig. 5E,E', cells positive for amph1 or amph2, respectively, were also stained by the HDC antibody. These results identify ECL cells as a new localization for amph1 and amph2 in the stomach.

We further investigated the subcellular distribution of amphiphysin in ECL cells using confocal microscopy. As shown in Fig. 6, both amph isoforms are found in the cytoplasm with bright spots at the plasma membrane.

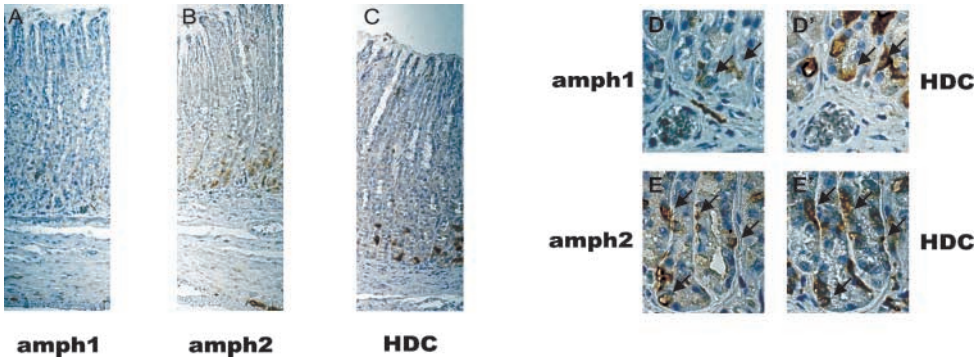


Fig. 5. Immunohistochemical detection of amph1, amph2, and histidine decarboxylase (HDC), the marker enzyme for ECL cells, in the rat stomach. All three antigens were detected in cells of the basal mucosa (A-C). Immunostaining of serial thin sections identified ECL cells as the amph1 and amph2 positive mucosa cells (D,E).

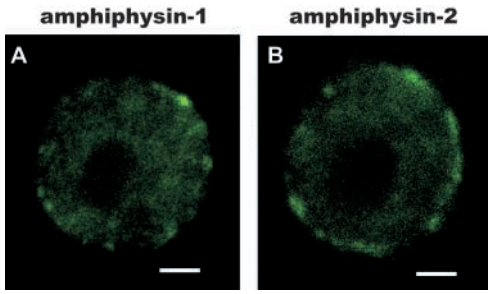


Fig. 6. Immunolocalization of amphiphysin isoforms in isolated ECL cells using subtype-specific antibodies. Both isoforms are found in the cytoplasm with bright spots at the plasma membrane. Bars, 2 μm.

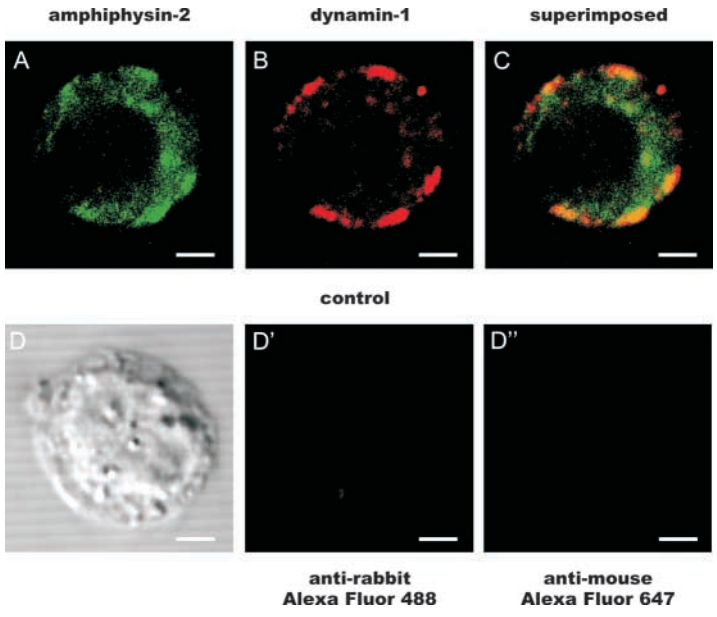
Colocalization of dynamin and amphiphysin

Double fluorescence immunocytochemistry was applied to investigate whether dynamin and amphiphysin colocalize. As shown in Fig. 7A-C, a partial colocalization could be observed at the plasma membrane. No staining could be detected in negative controls (Fig. 7D-D'').

Expression of amphiphysin mRNA in ECL cells

To investigate the presence of amphiphysin mRNA we performed RT-PCR with subtype-specific primers and RNA from highly enriched ECL cells. As shown in Fig. 8A, a 398 bp product could be amplified from ECL cell cDNA using primers specific for amph1 (lane 2). Compared with the positive control (rat brain cDNA, lane 3), the band obtained from ECL cell cDNA was less intensive when equal amounts of cDNA were used. No band was observed in negative controls (lane 4).

Fig. 8B shows the results obtained with primers specific for amph2. These primers include one of the alternative splice sites (the region encoding amino acids (aa) 174-204) of amph2. Two bands could be amplified from ECL cell cDNA: a weak band of 761 bp and a strong band of 668 bp (lane 2). In the positive control (rat brain cDNA, lane 3) the same bands could be observed, but with the opposite intensities. These results suggest that in ECL cells the splice variants of amph2 lacking aa 174-204 are more abundant than the splice variants including this part. Because several splice variants of amph2 have been described and found to be expressed in different tissues (Wigge et al., 1997), we amplified full-length amph2 from ECL cell cDNA to determine the splice variants present in these neuroendocrine cells. As shown in Fig. 8C, several bands were obtained (lane 2). Subsequent cloning and sequencing revealed the presence of mRNAs encoding the previously described rat amph2-1, amph2-5, amph2-6 and amph2-7/BIN1 along with a previously undetected splice form [for a comparison of rat amph2 splice variants see Fig. 1C in Wigge et al. (Wigge et al., 1997)]. This splice variant differs from full-length amph2-1 only in that it lacks the region encoding aa 174-204. In accordance to the nomenclature of Wigge et al. (Wigge et al., 1997), we name this splice form amph2-1b.



Interaction of dynamin and amphiphysin

To examine the interactions between amphiphysin and dynamin in vitro, co-immunoprecipitation was performed on total protein extracts of isolated, highly enriched ECL cells using the polyclonal amph2 antibody 1874 (Ramjaun et al., 1997). Brain extracts served as positive control. The immunoprecipitates were seperated by SDS-PAGE, blotted to PVDF membranes and incubated with antibodies against dyn1, amph1 and amph2. As shown in Fig. 9, dyn1, amph1

Fig. 7. Double immunolabeling of isolated ECL cells with antibodies against amph2 (A) and dyn1 (B). When images are superimposed, a partial colocalization at the plasma membrane can be observed (C). No staining is detectable in negative controls (secondary antibody only) (D-D''). Bars, 2 μm.

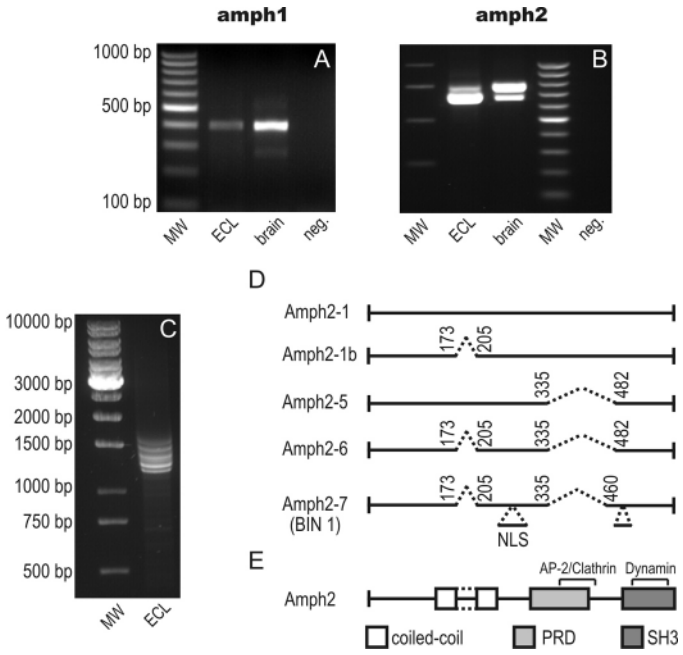


Fig. 8. Detection of amph1 (A) and amph2 (B) in ECL cells by RT-PCR. Positive (brain) and negative controls are also shown. Owing to the inclusion of an alternative spliced site, two bands were detected for amph2 (B). Amplification of full-length amph2 from ECL cell cDNA produced several bands, implying the presence of multiple splice variants (C). MW, molecular weight markers. Overview of amph2 splice forms found in ECL cells (modified from Wigge et al., 1997) (D). Domain structure of the amph2 gene product. Binding sites for AP-2, clathrin and dynamin are marked. PRD, proline rich domain; SH3, src homology domain (E).

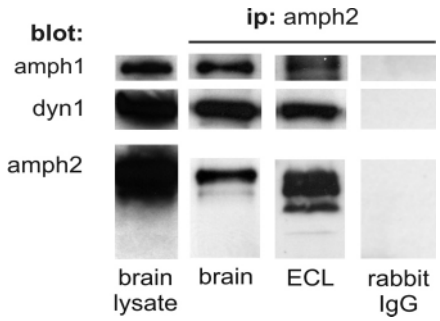


Fig. 9. Co-immunoprecipitation of amphiphysin and dynamin from total protein extracts from brain and highly enriched isolated ECL cells. Immunoprecipitation was performed with the amph2-antibody. The control-ip did not show any bands.

and amph2 were detected in immunoblots, confirming the interaction of dynamin and amphiphysin. Compared with brain, several smaller bands were detected by the anti-amph2 antibody in ECL cell extracts, indicating the presence of splice variants also detected by RT-PCR (see above). A negative control using unrelated rabbit IgGs did not produce any bands.

Discussion

In this study, we investigated the expression of dynamin and amphiphysin in gastric neuroendocrine ECL cells. We found that ECL cells express all dyn and amph isoforms and that these proteins interact in vitro. In addition, we observed a partial colocaliziation of dyn1 and amph2 at the plasma membrane. To our knowledge, this is the first report of the expression of all dynamin isoforms in a non-tumor cell. This observation supports the hypothesis that the different isoforms may have different functions (Urrutia et al., 1997).

Neuronal dyn1 is the best examined among dynamin isoforms. It participates in clathrin-dependent (Takei et al., 1995; Shupliakov et al., 1997; Hinshaw and Schmid, 1995) and clathrin-independent synaptic vesicle recycling (Artalejo et al., 1995; Daly et al., 2000), internalization and/or signal transduction of a variety of G protein-coupled receptors (GPCRs) (Gaborik et al., 2001; Whistler and von Zastrow, 1999; Daaka et al., 1998) and growth factor receptors (Scaife et al., 1994), and is also involved in neuronal growth (Torre et al., 1994). Like dyn1, ubiquitous dyn2 takes part in receptor-mediated endocytosis (Cao et al., 1998) and uptake of GPCRs (Szaszak et al., 2002). Furthermore, it is essential for internalization of caveolae (Henley et al., 1998; Oh et al., 1998), the release of vesicles from the trans-golgi network (Jones et al., 1998), regulation of actin assembly (Ochoa et al., 2000; McNiven et al., 2000b; Orth et al., 2002) and phagocytosis (Gold et al., 1999). It has also been shown to participate in hormone secretion in neuroendocrine cells (Yang et al., 2001).

Stimulation of ECL cells with gastrin and subsequent exocytosis of histamine leads to a reduction of the number of secretory vesicles, while the number of microvesicles increases (Zhao et al., 1999), suggesting compensatory vesicle membrane recycling as observed in other neuroendocrine cells (Schmid and Damke, 1995). Immunofluorescence microscopy in the current study revealed a distribution of dyn1 and dyn2 in the cell body as well as at the plasma membrane, suggesting a role for these isoforms in the recycling of secretory vesicles following histamine release. In chromaffin cells, which share many characteristics with ECL cells, two different forms of vesicle recycling have been described. Depending on the type of stimulation, dyn1-dependent, clathrin-independent rapid endocytosis and dyn2- and clathrin-mediated slow endocytosis could be observed (Artalejo et al., 2002). These two different modes of vesicle recycling could also exist in ECL cells.

Furthermore, a role of dyn1 in internalization and signal transduction of the G_q-coupled gastrin receptor or growth factor receptors seems probable. Binding of gastrin to the gastrin receptor in ECL cells of Mastomys natalensis leads to the activation of the mitogen-activated protein kinase pathway (Kinoshita et al., 1998). This process has been shown to be dynamin-dependent in several GPCRs (Whistler and von Zastrow, 1999; Daaka et al., 1998; Ahn et al., 1999).

Dyn2 may also participate in exocytosis of histamine-containing vesicles. In chromaffin cells, dyn2 was found to be associated with the membrane of purified secretory granules where it interacts with free syntaxin 1A and might thereby control granule trafficking at the plasma membrane (Galas et al., 2000). Furthermore, dyn2 plays a key role in controlling hormone secretion from mouse pituitary cells (Yang et al., 2001). However, these dynamin functions remain speculative at present.

The function of dyn3, which is found in testes, brain, heart

and lung (Nakata et al., 1993; Cook et al., 1996; Cao et al., 1998), is largely unknown. In brain it is found in the postsynaptic region where it interacts with Homer and the metabotropic glutamat receptor (Gray et al., 2003). In testes, dyn3 might be involved in spermatogenesis (Kamitani et al., 2002). In ECL cells, dyn3 shows a perinuclear distribution and seems to be expressed at a much lower level compared to dyn1 and dyn2. Its role remains to be determined.

Amph1 plays an important role in synaptic vesicle recycling (David et al., 1996), as well as in neurite outgrowth (Mundigl et al., 1998). In testis, amph1 has a potential role in spermatogenesis (Watanabe et al., 2001). The numerous splice variants of Amph2 have several functions. In brain, amph1 and amph2 form heterodimers and are responsible for clathrin-mediated endocytosis (Wigge et al., 1997; Di Paolo et al., 2002). Our results show that both isoforms are expressed in ECL cells. We found RNAs for full-length amph2-1 as well as the shorter splice variants amph2-5, amph2-6 and amph2-7/BIN1. In addition, we discovered a previously undescribed splice form that we call amph2-1b, in accordance with the nomenclature of Wigge et al. (Wigge et al., 1997). When compared with amph2-1, amph2-1b lacks the region encoding aa 174-204, also called the N-terminal insert domain (NTID). This part has a role in amphiphysin dimerization and membrane targeting (Ramjaun et al., 1999). The function of this new splice variant remains to be determined.

It appears that the splice variants lacking the central domain (which harbors the binding sites for clathrin and AP-2) produce stronger bands than full-length amph2 (Fig. 8C). This might imply that these shorter splice variants may be involved in clathrin-independent endocytosis or other functions.

The src homolgy domains of both amphiphysin isoforms bind to the proline-rich domain of dynamin (David et al., 1996; Grabs et al., 1997; Ramjaun et al., 1997). This interaction is important for the direction of dynamin to sites of endocytosis (Wigge and McMahon, 1998). Performing co-immunoprecipitation experiments on protein extracts of enriched ECL cells, we confirmed the interaction of dynamins and amphiphysins in vitro. In addition, dyn1 and amph2 colocalized partialy at the plasma membrane underlining the importance of this functional partnership for ECL cell function.

In summary, our current data clearly show the presence and interaction of dynamin and amphiphysin isoforms in the gastric ECL cell, a typical neuroendocrine cell; similar features may be true for other endocrine cells of the gut

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