Persistent activation of $G_s\alpha$ through limited proteolysis by calpain

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Treatment of rat pituitary GH_4C_1 cell membranes with calpain, a calcium-activated cysteine protease, increased adenylate cyclase activity, and this activity was inhibited by a calpain inhibitor, leupeptin. Calpain treatment potentiated the activity of guanosine 5′-[γ-thio]triphosphate (GTP[S]), but did not attenuate $MnCl_2$ action on adenylate cyclase, suggesting that calpain acted at the G-protein level, rather than directly on adenylate cyclase. This calpain stimulation of adenylate cyclase was inhibited by an antibody raised against the C-terminal portion of $G_s\alpha$, but not by anti-$G_i2\alpha$ or anti-$G\beta$ antibodies. Furthermore, it was shown that $G_s\alpha$ is more susceptible to calpain-mediated proteolysis than $G_i2\alpha$ or $G\beta$. Therefore the stimulatory effect of calpain on adenylate cyclase is due to the cleavage of $G_s\alpha$ in GH_4C_1 cell membranes. Proteolysis of $G_s\alpha$ by μ-calpain involved sequential cleavages at two sites, resulting in the generation of a 39 kDa fragment first, and then a 20 kDa fragment, from the C-terminus. Treatment of GH_4C_1 cell membranes with cholera toxin increased the rate of cleavage. Cholera toxin treatment of intact GH_4C_1 cells induced the translocation of calpain from the cytosol to the membranes, a hallmark of calpain activation. In addition, treatment of intact GH_4C_1 cells with a calpain-specific inhibitor, benzyloxycarbonyl-Leu-leucinal, blocked the increased cAMP production and the down-regulation of $G_s\alpha$, which were produced by cholera toxin or pituitary adenylate cyclase-activating polypeptide. These results suggest that calpain sustains adenylate cyclase in an active form through the cleavage of $G_s\alpha$ to an active $G_s\alpha$ fragment. This is a novel calpain-dependent activation mechanism of $G_s\alpha$ and, thus, of adenylate cyclase in rat pituitary cells.

Key words: adenylate cyclase, cAMP, cholera toxin, pituitary adenylate cyclase-activating polypeptide (PACAP), protease.

INTRODUCTION

Heterotrimeric G-proteins composed of α, β and γ subunits play important roles in determining the specificity and temporal characteristics of the cellular responses to signals [1]. Many subtypes of G-proteins are present in individual cells, and transmit signals from receptors to adenylate cyclase, phospholipase C (PLC) or ion channels. It has been shown that prolonged agonist exposure often induces selective down-regulation of the α subunits of G-protein to which the receptor is normally coupled [2–4]. Chang and Bourne also showed that cholera toxin treatment induced a pronounced degradation of $G_s\alpha$ in rat pituitary GH_3 cells [5]. They provided a strong indication that cholera toxin treatment increases the susceptibility of $G_s\alpha$ to cleavage by endogenous protease(s). Thus it is possible that, on direct or indirect activation of G-proteins, α subunits are cleaved by intracellular protease(s) to be down-regulated.

Calpain is a Ca^{2+}-dependent cysteine protease that binds to membranes upon activation and proteolyses substrate proteins in a limited manner [6]. Two calpain species are known to exist ubiquitously: a form that is highly sensitive to Ca^{2+} (μ-calpain), and a form that is only slightly sensitive to this cation (m-calpain). Although the majority of calpain is located in the cytosol of cells, an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) induces calpain translocation to the plasma membrane or the cytoskeleton. Calpain can carry out the limited proteolysis of cytoskeletal proteins, as well as the proteolytic modification of proteins associated with multiple signalling cascades, such as protein kinase C [7], PLC [8], $pp60^{src}$ [9] and focal adhesion kinase [10]. Thus calpain is implicated in signal-transduction pathways, as well as cytoskeletal reorganization in response to elevations in $[Ca^{2+}]_i$. However, evidence for the involvement of calpain in signal transduction via the generation of cAMP is very scarce. It has been shown that basal and hormonal responsive levels of adenylate cyclase in platelet membranes from hypertensive rats are stimulated in a Ca^{2+}-dependent and leupeptin-sensitive manner [11]. This report strongly suggests that calpain is involved in a cAMP-generation system through the signal transduction of heterotrimeric G-protein-coupled receptors.

In the present paper, we elucidated the effects of calpain on the cAMP-generation system through the signal transduction of heterotrimeric G-protein-coupled receptors in rat anterior pituitary tumour GH_4C_1 cells. We found that treatment of GH_4C_1 cell membranes with calpain increased cAMP production and correlated proteolysis *in vitro* of the α subunit of G_s. Moreover, these effects were confirmed in intact GH_4C_1 cells using a calpain-specific cell-permeant inhibitor, benzyloxycarbonyl-Leu-leucinal (ZLLal). The results indicate that $G_s\alpha$ is indeed cleaved by calpain upon pituitary cell activation by a physiological agonist, pituitary adenylate cyclase-activating polypeptide (PACAP). To our knowledge, this is the first report of proteolytic activation of $G_s\alpha$ by calpain that suggests the presence of a novel regulation mechanism in the signal transduction of the cAMP-generation system in GH_4C_1 cells.

MATERIALS AND METHODS

Reagents

Drugs and chemicals were obtained from the following sources: ZLLal, leupeptin and PACAP were from Peptide Institute, Inc. (Osaka, Japan), guanosine 5′-[γ-thio]triphosphate (GTP[S]) and creatine kinase were from Boehringer Mannheim (Mannheim,

Abbreviations used: PLC, phospholipase C; ($[Ca^{2+}]_i$), intracellular calcium concentration; ZLLal, benzyloxycarbonyl-Leu-leucinal; PACAP, pituitary adenylate cyclase-activating polypeptide; GTP[S], guanosine 5′-[γ-thio]triphosphate; IBMX, 3-isobutyl-1-methylxanthine.

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Germany), and cholera toxin was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). RO20-1724 was a gift from Roche Co. (Tokyo, Japan). RM-1, AS-7 and SW-1 antisera against the G-protein subunits $G_s\alpha$, $G_i2\alpha$ and $G\beta$ respectively were obtained from New England Nuclear (Boston, MA, U.S.A.). The cAMP kit for radioimmunoassays was purchased from Yamasa Co. (Noda, Japan). Calpains were purified from bovine lung to homogeneity, as described previously [12].

Calpain treatment of the GH_4C_1 cell membrane

GH_4C_1 cells were a gift from Dr. Naohide Yamashita in the Fourth Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Japan. GH_4C_1 cells were grown in Ham's F10 medium (Gibco Laboratories, Tokyo, Japan) supplemented with 2.5% (v/v) fetal-calf serum (Gibco Laboratories) and 15% (v/v) horse serum [13]. GH_4C_1 cell membranes were prepared as described previously [14]. Briefly, the cells were harvested in PBS, pH 7.2, by centrifugation. The cell pellets were resuspended and stored for 15 min in an ice-cold 20 mM Tris/HCl solution, pH 7.6, containing 1 mM $MgCl_2$ and 2 mM EGTA. This suspension was then homogenized by 15 strokes in a Dounce glass homogenizer with pestle B. After the removal of undisrupted cells and nuclei by a 5-min centrifugation (100 ***g***, 4 °C), the supernatant was centrifuged at 4000 ***g*** for 15 min at 4 °C to obtain the membrane-rich fraction. This fraction was suspended in 5 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol, 1 mM EGTA and 10% (v/v) glycerol, and then stored at −80 °C until use. The cell membranes were washed once with TEN buffer [20 mM Tris/HCl (pH 7.6)/1 mM EDTA/25 mM NaCl] and centrifuged at 10000 ***g*** for 10 min. The membrane-rich fraction resuspended in TEN buffer at 0.1 mg/ml protein was treated with various concentrations of μ- or m-calpain in the presence of 2.5 mM $CaCl_2$, 28 mM 2-mercaptoethanol and 50 mM Tris/HCl, pH 7.5, for 30 min at 30 °C. The reaction was terminated by the addition of 1 mM leupeptin, and subjected to immunoblot analysis or the adenylate cyclase assay.

For the adenylate cyclase assay, calpain-treated or control membranes (0.5 mg/ml) containing 2.5 mM Ca^{2+} were diluted to 0.5 mM Ca^{2+}, and the adenylate cyclase assay mixture [75 mM Tris/HCl (pH 7.5)/5 mM phosphoenolpyruvate/3 μg/ml pyruvate kinase/0.2 mM RO20-1724 (phosphodiesterase inhibitor)/1 mM ATP/2 mM $MgCl_2$/1 mM EGTA/0.33 mM dithiothreitol/50 μg/ml BSA] was added in the presence or absence of 10 μM GTP[S] or 1 mM $MnCl_2$. Another phosphodiesterase inhibitor, 1 mM 3-isobutyl-1-methylxanthine (IBMX), was used in place of RO20-1724 in the adenylate cyclase assay when the effects of RO20-1724 and IBMX on calpain activity were to be compared. The mixtures were incubated for 20 min at 37 °C, and the reaction was terminated by addition of 0.3 M HCl. The generated cAMP was determined by radioimmunoassay using the Yamasa cAMP ^{125}I-assay kit.

[^{32}P]ADP-ribosylation of GH_4C_1 cell membranes (0.1 mg/ml protein) by cholera toxin was performed essentially as described previously [14]. [^{32}P]ADP-ribosylated membranes were treated with 4 units/ml μ- or m-calpain, as previously described. The reactions were stopped by the addition of ice-cold trichloroacetic acid to a final concentration of 10% (w/v), and the mixtures were centrifuged (12000 ***g***, 4 °C) for 10 min. The resulting pellets were subjected to electrophoresis in SDS/10% polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue, dried and then exposed to Phosphor storage-screen autoradiography using a Fujix Bio-Imaging Analyzer, according to the manufacturer's instructions. The resultant pellets were also subjected to immunoblot analysis for $G_s\alpha$, as described previously. The results presented are representative of three or more experiments performed under identical conditions. Statistical comparisons were made by Student's *t* test analysis.

For the immunoblot analysis, calpain-treated or control membranes (0.5–5 μg of protein) were subjected to SDS/PAGE [15] using 10% (w/v) acrylamide gels, transferred to the PVDF membrane (Millipore, Bedford, MA, U.S.A.), and blocked with Tris-buffered saline [20 mM Tris/HCl (pH 7.5)/150 mM NaCl] containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat skimmed milk. The membranes were incubated overnight with the primary antibodies, anti-$G_s\alpha$ or anti-$G_i2\alpha$ (1:250 dilution), at 4 °C, washed four times with Tris-buffered saline, and probed with horseradish-peroxidase-conjugated goat anti-rabbit antibody (1:1000 dilution). Sought-after protein was revealed using an enhanced chemiluminescence (ECL) Western blot detection system (Amersham, Arlington, IL, U.S.A.) according to the manufacturer's protocol. The blots were analysed using an ACI Imagiepro computer-associated video-image analysis system (ACI Japan, Kanagawa, Japan). It was confirmed that the labelling intensity of each band was directly proportional to the amount of membrane protein from 0.5–3.0 μg for RM-1 $G_s\alpha$ antiserum, and from 10–48 μg for SW-1 $G_i2\alpha$ antiserum.

Translocation of calpains in GH_4C_1 cells

Cells were stimulated with 2 μg/ml cholera toxin for 0, 15, 30, 60, 90 or 120 min, or stimulated with 0.2 μM PACAP for 0, 15, 30, 60 or 120 min, and cells were washed twice with PBS containing 1 mM EDTA. Freeze–thaw lysis was accomplished by following the methods of Wedegaertner et al. [16]. Briefly, PBS-washed plates were placed in a solid-CO_2/ethanol bath for 2 min and stored at −80 °C. Plates were transferred to a 37 °C water bath, and cells were thawed in 0.8 ml of 0.5 M NaCl lysis buffer [20 mM Hepes (pH 7.2)/0.5 M NaCl/2.5 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol/0.5 mM PMSF/0.1 mM leupeptin]. Broken cells were rapidly scraped and transferred to a centrifuge tube. This suspension was then centrifuged at 150000 ***g*** for 20 min in a TL-100 ultracentrifuge. The supernatant was designated as the 'cytosol fraction'. The pellets were then lysed with 0.4 ml of RIPA buffer [1% (v/v) Triton X-100/20 mM Tris/HCl (pH 7.5)/150 mM NaCl/2 mM EDTA/0.5 mM PMSF/0.1 mM leupeptin] for 15 min on ice, and centrifuged at 15000 ***g*** for 5 min. This further supernatant was designated as the 'membrane fraction'. These samples were added to SDS sample buffer, subjected to SDS/PAGE on 10% (w/v) polyacrylamide gels, and immunostained with anti-calpain or anti-$G_s\alpha$ antibodies. Exposed 80-kDa calpain bands were scanned and analysed densitometrically by using NIH Image 1.6 software (National Institutes of Health, Bethesda, MD, U.S.A.).

Calpain inhibitor treatment of intact GH_4C_1 cells

GH_4C_1 cells (1×10^5/dish) were placed in a 35-mm dish, and cultured for 4 days. The cells were preincubated for 1 h with Ham's F12 medium, containing 20 mM Hepes, pH 7.2, 1 mg/ml BSA and various concentrations of a calpain inhibitor (ZLLal) or a vehicle (0.1% DMSO). The cells were then stimulated with 2 μg/ml cholera toxin or 0.2 μM PACAP for 8 h. To determine the levels of intracellular cAMP, the medium was removed, and intracellular cAMP was extracted with 0.8 ml of 0.3 M HCl for 10 min at room temperature. Samples were centrifuged at 10000 ***g*** for 3 min at 2 °C, and the amount of cAMP in the supernatant

was determined by cAMP radioimmunoassay. At the same time, the pellets of HCl-treated cells were solubilized in SDS sample buffer, neutralized by the addition of NaOH and subjected to immunoblot analysis, and the membrane levels of $G_s\alpha$ were then determined.

RESULTS

Increase in cAMP production in GH_4C_1 cell membranes on calpain treatment

In order to determine whether calpain influences the cAMP generation system in rat pituitary GH_4C_1 cells, GH_4C_1 cell membranes were treated with μ-calpain and subjected to an assay of adenylate cyclase. As shown in Figure 1(A), left panel, calpain treatment increased cAMP production in GH_4C_1 cell membranes 3.7-fold compared with untreated membranes. This increase was inhibited by the presence of an *in vitro* calpain inhibitor, leupeptin, during calpain treatment of the membranes, suggesting that the effect was due to calpain. The increase in cAMP production of the membranes treated with calpain was amplified to 3.8-fold of the control by GTP[S], an unhydrolysable homologue of GTP (Figure 1A, centre panel). However, there was no significant difference in cAMP production in these membranes when the direct adenylate cyclase activator, $MnCl_2$, was added to the adenylate cyclase assay mixture (Figure 1A, right panel). These results demonstrated that substrates of calpains in the GH_4C_1 cell membranes other than the catalytic unit of adenylate cyclase, possibly the G-protein subunit(s), are responsible for the adenylate cyclase activation. Figure 1(B) shows that the optimal concentration was approx. 2.5 units/ml μ-calpain, and the effects observed at its higher concentrations were probably due to the extensive degradation of the component for adenylate cyclase activation. Furthermore, calpain treatment in the presence of GTP[S] caused the higher production of cAMP compared with that in the absence of GTP[S] with 2.5 units/ml μ-calpain activity: the cAMP production was 0.7 pmol/mg per min in the control membranes, and 1.9 pmol/mg per min in membranes incubated with GTP[S] (Figure 1B). These results indicate that GTP[S] enhances the stimulation of adenylate cyclase in GH_4C_1 cell membranes. To avoid the possibility that phosphodiesterases are inactivated by calpains, with the consequent abolition of cAMP degradation, the assay was performed in the presence of phosphodiesterase inhibitors IBMX (1 mM), RO20-1724 (0.2 mM), or neither of them. Neither inhibitor influenced the increase of cAMP production by calpain (results not shown), suggesting that the action of calpain is due to an increase in cAMP generation, rather than a decrease in cAMP degradation.

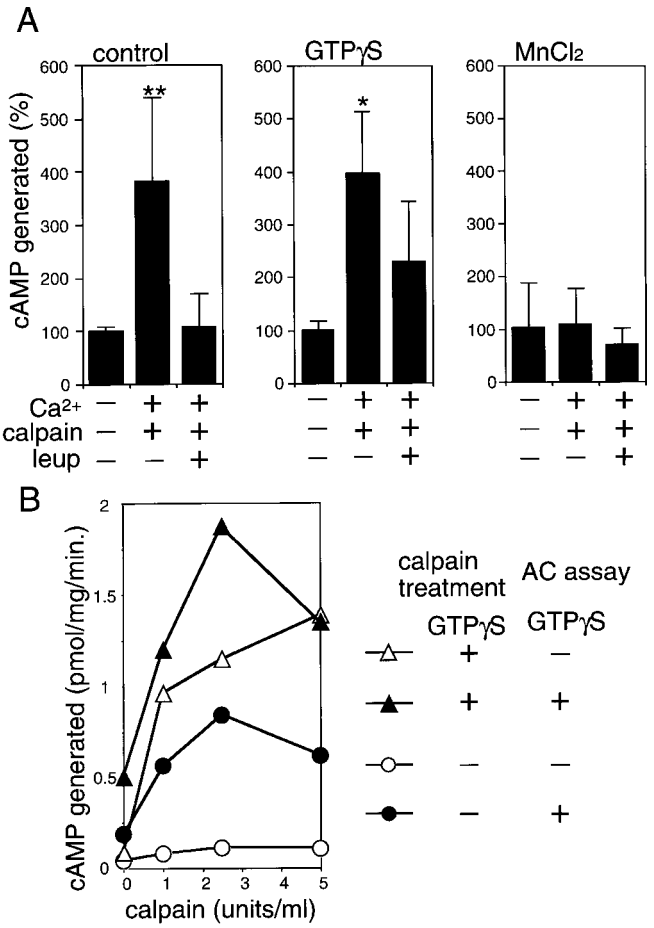

Figure 1 Effect of μ-calpain on adenylate cyclase activity of GH_4C_1 cell membranes

(**A**) Cell membranes (0.1 mg/ml protein) were treated in the presence or absence of 2.5 mM $CaCl_2$ (Ca^{2+}), 5 units/ml of μ-calpain (calpain) or 1 mM leupeptin (leup) for 30 min at 30 °C, as described in the Materials and methods section. Subsequently, calpain-treated membranes were incubated with an adenylate cyclase (AC) assay mixture in the presence or absence of 10 μM GTP[S] (GTPγS) or 1 mM $MnCl_2$. cAMP thus generated was determined by radioimmunoassay. Data are expressed as the percentage of activity observed in the control membranes. Means $\pm$ S.E.M. for the absolute values for control activities were found to be 17.6, 266.7 and 55.2 pmol/min per mg of protein for basal, GTPγS and $MnCl_2$ stimulation respectively. The graphs are representative of three experiments with comparable results. *, $P < 0.05$; **, $P < 0.01$ as measured against control levels. (**B**) Cell membranes were treated with various concentrations of μ-calpain in the presence or absence of 10 μM GTPγS, and the activity of adenylate cyclase was determined in the presence or absence of 10 μM GTPγS.

Identification of $G_s\alpha$ as the molecule responsible for the increased cAMP production on calpain treatment of GH_4C_1 cell membranes

The G-protein subunits involved in the regulation of adenylate cyclase activity are $G_s\alpha$ (stimulative), $G_i\alpha$ (inhibitory) and $G\beta\gamma$ (inhibitory or stimulative) [17]. Therefore we examined which subunit of the G-proteins was responsible for the calpain proteolysis that increases cAMP production. The calpain-treated membranes were preincubated with antibodies raised against the C-terminal portions of $G_s\alpha$, $G_i2\alpha$ and $G\beta$ before the assay of adenylate cyclase (Figure 2). The stimulatory effect of calpain treatment on cAMP production was unaffected by normal rabbit serum (IgG). Preincubation with the $G_s\alpha$ antibody before adenylate cyclase assay suppressed the stimulation to 58% of control values. However, membranes preincubated with antibodies against $G_i2\alpha$ and $G\beta$ showed no effect on the stimulation (Figure 2). These data suggest that proteolysis of $G_s\alpha$ by calpain, at least, caused the activation of adenylate cyclase.

Cleavage of G-protein subunits in GH_4C_1 cell membranes by calpains

We then examined the cleavage of $G_s\alpha$, $G_i2\alpha$ and β subunits in GH_4C_1 cell membranes by purified calpains, and detected the degradation products by Western blotting using antibodies that recognized their C-termini (Figure 3). There are two forms of $G_s\alpha$ in GH_4C_1 cell membranes with apparent molecular masses of 52 kDa and 45 kDa, of which 52 kDa is the major form of the $G_s\alpha$ protein detected on Western blots. As shown in Figure 3(A), both of them were degraded by μ-calpain to 51.2% (52 kDa) and

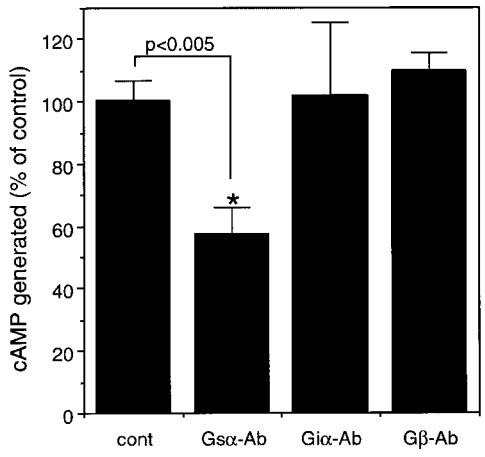

Figure 2 Effects of anti-$G_s\alpha$, anti-$G_i2\alpha$ or anti-$G\beta$ antibodies on adenylate cyclase activity in μ-calpain-treated GH_4C_1 cell membranes

Cell membranes treated with 2 units/ml calpain as described in the legend to Figure 1 were incubated with 0.2 mg/ml of non-immune rabbit serum IgG (cont) or rabbit polyclonal antibodies (Ab) raised against the C-terminal portion of $G_s\alpha$, $G_i2\alpha$ or $G\beta$ (1:40–80 dilutions) for 20 min at 20 °C, and subjected to adenylate cyclase assay, as described in the Materials and methods section. Experiments were performed in triplicate. Data are expressed as the percentages of cAMP generation in rabbit IgG-treated membranes; values shown are means ± S.E.M. for three independent experiments performed in duplicate.

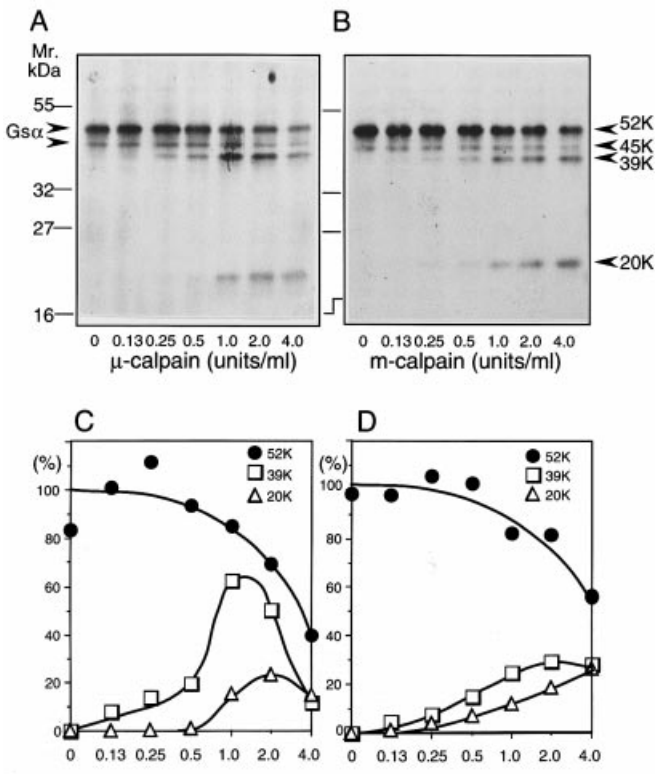

Figure 3 Dose-dependent cleavage of $G_s\alpha$ in GH_4C_1 cell membranes by calpains

Cell membranes (0.1 mg/ml) were treated with the indicated concentrations of μ-calpain (**A**) or m-calpain (**B**) as described in the Materials and methods section, subjected to SDS/PAGE on 10% (w/v) polyacrylamide gels, and immunoblotted with RM-1 as the primary antibody. Densitometric tracings of the results are shown in (**C**) for (**A**), and in (**D**) for (**B**). Data for three individual experiments yielded similar results.

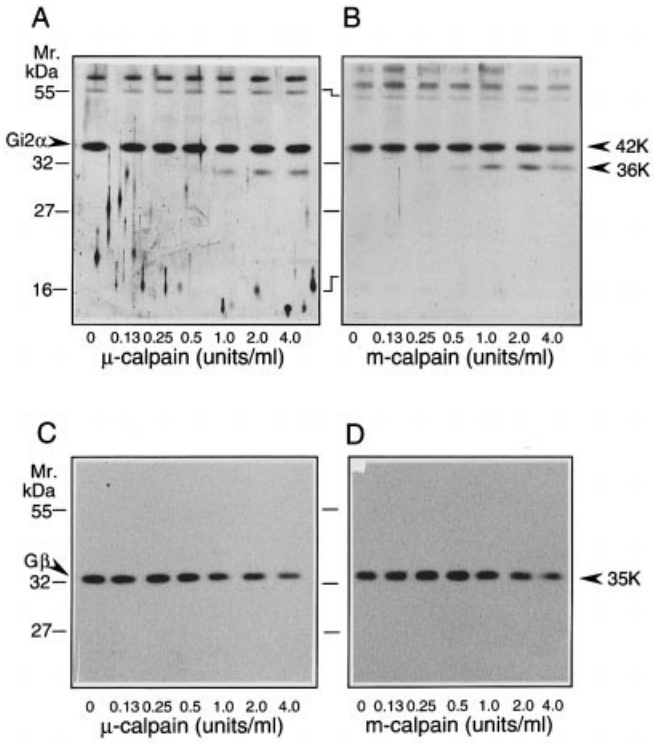

Figure 4 Dose-dependent cleavage of $G_i2\alpha$ and $G\beta$ in GH_4C_1 cell membranes by calpain

Cell membranes (0.1 mg/ml protein) were treated with indicated concentrations of μ-calpain (**A**, **C**) or m-calpain (**B**, **D**) as described in Figure 3, subjected to SDS/PAGE in 10% polyacrylamide gels, and immunoblotted with antibodies raised against the C-terminal region of $G_i2\alpha$ (**A**, **B**) or $G\beta$ (**C**, **D**) as the primary antibody. Representative immunoblots are shown; three separate experiments gave similar results.

43.7% (45 kDa) of control values at a concentration of 4 units/ml, and generated sequentially an immunoreactive 39 kDa, and then 20 kDa, form in a dose-dependent manner (Figure 3C). It seemed that the same C-terminal fragments were generated from both the 52 kDa and 45 kDa forms of $G_s\alpha$. The amount of μ-calpain required for the maximal generation of the 39-kDa fragment was 1–2 units/ml, which was almost identical with the optimal amount needed for the activation of adenylate cyclase activity by μ-calpain treatment of the GH_4C_1 cell membranes, as described previously (Figure 1B). A similar degradation also occurred by m-calpain, although with less potency than μ-calpain (Figures 3B and 3D). On the other hand, $G_i2\alpha$ (42 kDa) was degraded by only 20% compared with control values by μ-calpain at 4 units/ml, and this generated an immunoreactive 36-kDa fragment by both μ- and m-calpains dose-dependently (Figures 4A and 4B). As shown in Figures 4(C) and 4(D), calpain (4 units/ml) also cleaved a lesser amount of β subunit (29% of control) of the GH_4C_1 cell membranes. These results indicate that calpain cleaved $G_s\alpha$ more effectively than $G_i2\alpha$ or the β subunit.

Preferential cleavage of activated $G_s\alpha$ by calpain

In the present experiment, the generation of cAMP was enhanced to a much greater extent when GTP[S] was present during

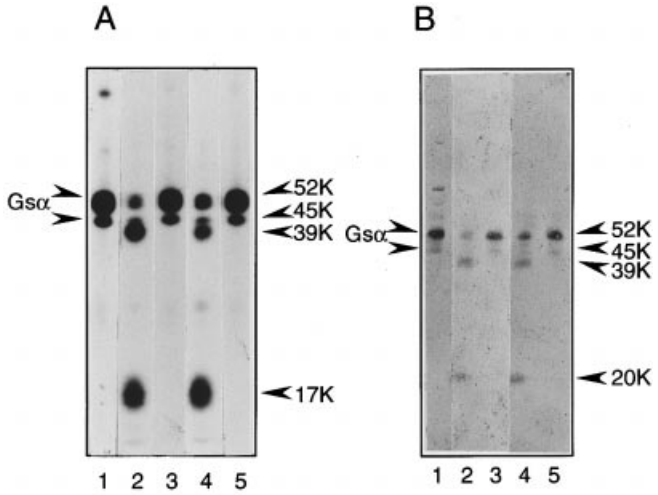

Figure 5 Effects of ADP-ribosylation on the cleavage of $G_s\alpha$ by calpain

$G_s\alpha$ in GH_4C_1 cell membranes was labelled with [^{32}P]ADP by cholera toxin (lane 1) and cleaved by 4 units/ml μ-calpain (lanes 2 and 3) or m-calpain (lanes 4 and 5) in the presence (lanes 3 and 5) or absence (lane 2 and 4) of 1 mM leupeptin. All reaction mixtures were analysed by SDS/PAGE. (**A**) Autoradiogram of the dried gels is shown; (**B**) immunoblot analysis of $G_s\alpha$. The masses of $G_s\alpha$ forms pre- and post-treatment with calpain are shown in kDa to the right of the gels.

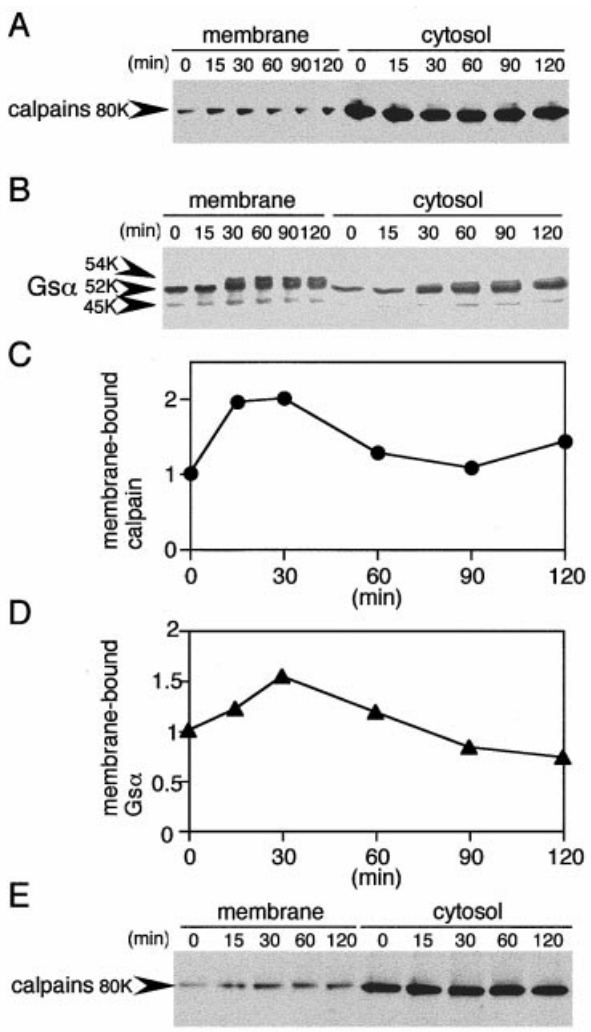

Figure 6 Translocation of calpains from the cytosol to the membrane in GH_4C_1 cells treated with cholera toxin or PACAP

Cells were stimulated with 2 μg/ml cholera toxin for 0, 15, 30, 60, 90 or 120 min (**A**–**D**), or with 0.2 μM PACAP for 0, 15, 30, 60 and 120 min (**E**). The cells were washed and broken by freeze–thaw lysis using solid CO_2/ethanol. The broken cells were pelleted by ultracentrifugation at 150000 ***g*** for 20 min. The pellets (membrane and nuclear fractions) were lysed with RIPA buffer containing 1% (w/v) Triton X-100, centrifuged at 15000 ***g*** for 5 min, and supernatant was regarded as the membrane protein fraction. The supernatants obtained by ultracentrifugation (cytosol) and RIPA-soluble fraction of particulate (membrane) from equal cell number were subjected to SDS/PAGE on 10% gels, and immunoblotted with anti-calpains (**A**, **E**) or anti-$G_s\alpha$ (**B**) antibodies. The immunoblot results shown in (**A**) and (**B**) were densitometrically quantified as the relative amounts of membrane-bound calpains (●) or $G_s\alpha$ (▲) in (**C**) and (**D**). Two separate experiments gave similar results.

calpain treatment of GH_4C_1 cell membranes, suggesting that the active form of $G_s\alpha$ is more sensitive to calpain (Figure 1B). Cholera toxin, in contrast with hormonal stimulation, directly activates $G_s\alpha$ by ADP-ribosylation, leading to stimulation of adenylate cyclase. Therefore we examined the effects of cholera toxin on the calpain proteolysis of $G_s\alpha$. As shown in Figure 5(A), ADP-ribosylated $G_s\alpha$ was cleaved drastically to 13% (52 kDa) and 30% (45 kDa) of the control values by μ-calpain at 4 units/ml, and almost similar susceptibility was shown towards m-calpain. These degradations were completely blocked by leupeptin. The generated fragments detected by autoradiography of [^{32}P]ADP-ribose labelling at Arg-201 in the central portion of $G_s\alpha$ were 39 kDa and 17 kDa in terms of molecular mass, showing again that calpain cleaved $G_s\alpha$ at two interdomain sites. Moreover, we tested the same digested samples by immunoblot analysis of $G_s\alpha$ (Figure 5B). For both the 52-kDa and 45-kDa forms of $G_s\alpha$, the α subunits were degraded to 15% of the control values, which were more prominent values compared with non-ADP-ribosylated $G_s\alpha$ in the membranes, and generated similar 39-kDa and 20-kDa fragments to those shown in Figure 3(A).

Translocation of calpain from the cytosol to membrane fractions of GH_4C_1 cells

The experiments using cell membranes indicated the involvement of calpain in the $G_s\alpha$ degradation and the potentiation of adenylate cyclase. We therefore examined the translocation of calpain, which is an important indicator of calpain activation, in intact GH_4C_1 cells. Cells were stimulated with cholera toxin, lysed by the freeze–thaw method using solid CO_2/ethanol, separated into cytosol and membrane fractions by ultracentrifugation (at 150000 ***g*** for 20 min), and these fractions were then subjected to SDS/PAGE and immunoblot analysis. As shown in Figures 6(A) and 6(C), immunoblot analysis of the insoluble fractions using an anti-calpain monoclonal antibody (1D10A7), which recognized μ- and m-calpain large subunits (80 kDa), revealed a reproducible increase in the levels of membrane calpains within 30 min of stimulation. On the other hand, cholera toxin modified $G_s\alpha$ from a 52-kDa intact form to a 54-kDa ADP-ribosylated one after 30 min of incubation, and the amount of the membrane-bound $G_s\alpha$ was then degraded to 73% ($n = 2$) of both 54-kDa and 52-kDa forms of $G_s\alpha$ after 2 h of incubation (Figures 6B and 6D). We found also that cholera toxin increased the amount of soluble $G_s\alpha$ in the cytosolic fraction by 1.54-fold ($n = 2$) of control $G_s\alpha$ in the non-stimulated cytosolic fraction after 1 h of incubation, which is similar to results obtained by

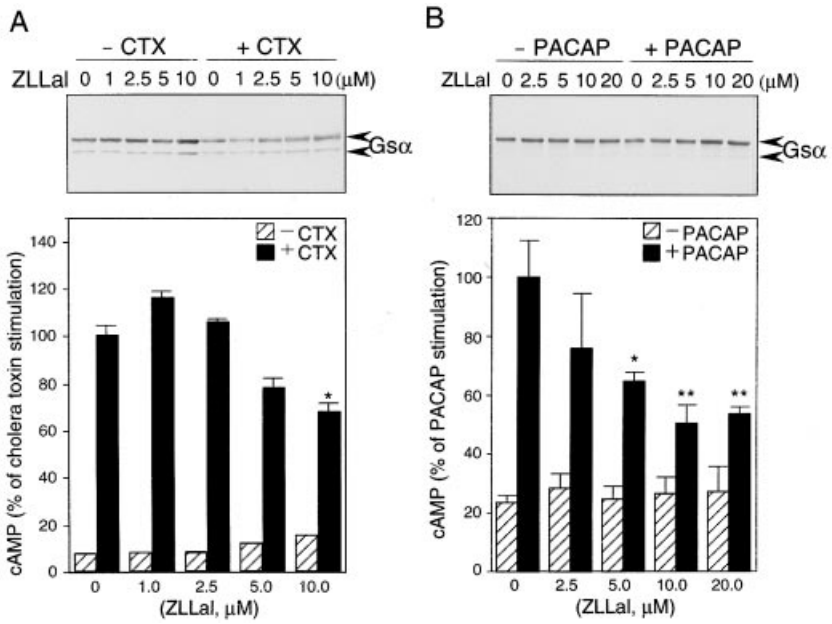

Figure 7 Effect of a calpain inhibitor ZLLal on cAMP levels in GH_4C_1 cells treated with cholera toxin (CTX) or PACAP

Cells were preincubated with ZLLal for 1 h at the indicated concentrations, and stimulated with (black bars) or without (hatched bars) 2 μg/ml CTX (**A**) or 0.2 μM PACAP (**B**) for 8 h. Intracellular cAMP levels (lower panels) were determined as described in the Materials and methods section. The residues of the cells extracted with 0.3 M HCl were solubilized in Laemmli's SDS sample buffer, subjected to SDS/PAGE and immunoblotted with anti-$G_s\alpha$ antibody (upper panels). Experiments for cAMP determination were performed in triplicate in four separate experiments, and gave similar results. $^*P < 0.05$ and $^{**}P < 0.01$, as measured against control levels.

Levis and Bourne [21]. In addition, the more physiologically relevant stimulant, PACAP, also induced a reproducible increase in membrane calpains within 30 min of stimulation (Figure 6E). This indicated that cholera toxin or PACAP treatment induced the translocation of calpain from the cytosol to the membranes and the subsequent degradation of membrane-bound active $G_s\alpha$ in the GH_4C_1 cells.

ZLLal blocks $G_s\alpha$ degradation induced by cholera toxin or PACAP

We have demonstrated previously that ZLLal is specific for calpain in relation to the two major intracellular cytoplasmic proteases, calpain and proteasome [18]. We examined the effect of ZLLal on $G_s\alpha$ degradation induced by the cholera-toxin treatment of intact GH_4C_1 cells (Figure 7). Treatment of cells with cholera toxin (2 μg/ml, 8 h) reduced the membrane levels of $G_s\alpha$ to 23% of control values without cholera toxin, but the intracellular cAMP level remained high (Figure 7A); these results are similar to those of Chang and Bourne [5]. Cells were pre-treated with ZLLal for 60 min, followed by addition of cholera toxin. In the absence of cholera toxin, ZLLal at a concentration of 10 μM, which is sufficient to abolish calpain activity, did not attenuate the significant production of cAMP in GH_4C_1 cells after treatment for 9 h. However, ZLLal inhibited the cholera toxin-induced degradation of membrane $G_s\alpha$ dose-dependently, and increased the amount of $G_s\alpha$ to 3.5-fold of control levels at 10 μM concentration (Figure 7A, upper panel). In addition, ZLLal decreased the sustained cAMP production by cholera toxin to 70% of control values at 10 μM (Figure 7A, lower panel). Furthermore, the inhibition by ZLLal of both the increase in cAMP production and the degradation of membrane $G_s\alpha$ was also observed when the intracellular $G_s\alpha$ was activated by a more physiological stimulus, PACAP; the cAMP production in GH_4C_1 cells treated with PACAP was reduced to 50% of the control level at 10 μM ZLLal (Figure 7B). The inhibitory effect of ZLLal on cAMP accumulation produced by cholera toxin or PACAP was not altered by the phosphodiesterase inhibitor IBMX (1 mM; results not shown). Thus the present results suggest that calpain is involved in the cholera toxin or PACAP effects on GH_4C_1 cells, and the ability of calpain to stimulate cAMP accumulation results from direct activation of the $G_s\alpha$/adenylate cyclase pathway, rather than inhibition of phosphodiesterase activity.

DISCUSSION

We have demonstrated that the calcium-sensitive protease, calpain, stimulated cAMP generation in rat pituitary GH_4C_1 cells. We previously reported that GH_4C_1 cells express μ- and m-calpain [19]. In the present experiments, cholera toxin and PACAP stimulated the translocation of calpain from the cytosol to the membrane fractions, as shown in Figure 6. It is possible that calpain could be involved in the signal-transduction system in rat pituitary GH_4C_1 cells.

Interestingly, the adenylate cyclase activity in GH_4C_1 cell membranes was stimulated with calpain treatment. To examine which activity of adenylate cyclase or trimeric G-protein was modified by calpain, Ca^{2+}/calpain-treated membranes were stimulated with GTP[S] or $MnCl_2$. It is known that GTP[S] and $MnCl_2$ activate the α subunits of the G-protein and adenylate cyclase respectively. The activity of adenylate cyclase in Ca^{2+}/calpain-treated membranes was not modified by $MnCl_2$ (Figure 1A, right panel), indicating that neither calpain nor $CaCl_2$ affected adenylate cyclase activity directly. Since the $G_s\alpha$ in the GH_4C_1 cells links its effectors to adenylate cyclase types II and IV, the activities of which are independent of the

Ca^{2+}–calmodulin complex [20], the increase in adenylate cyclase activity cannot be due to the presence of Ca^{2+} in calpain treatment. More clearly, the stimulation in adenylate cyclase activity by calpain treatment was inhibited by the addition of leupeptin or anti-$G_s\alpha$ antibody, suggesting that the adenylate cyclase activation was caused by direct action of calpain on $G_s\alpha$. In fact, calpain prefers the α subunit of G_s to either the $G_i2\alpha$ or β subunits as its substrate (Figures 3 and 4). In addition, cholera toxin treatment increased the rate of $G_s\alpha$ cleavage by calpain in GH_4C_1 cell membranes (Figure 5A), suggesting that an alteration in the conformation of $G_s\alpha$ by cholera toxin facilitated proteolysis. This is consistent with the suppositions that any type of activation of $G_s\alpha$ by toxin-catalysed ADP-ribosylation, GTP[S] or agonist activation accelerates its degradation [21,22]. The data showing the proteolysis of $G_s\alpha$ by μ-calpain rather than m-calpain were anticipated because μ-calpain, which requires lower calcium concentrations for activation *in vitro*, has been considered as the more probable candidate for intracellular proteolytic phenomena (Figure 3).

We demonstrated that calpain proteolysed $G_s\alpha$ first to the 39-kDa fragment, and then to the 20-kDa one, by using SDS/PAGE, and activated adenylate cyclase in the GH_4C_1 membranes (Figures 1 and 3). According to a recent study on structure and function [23], the α subunit of heterotrimeric G-proteins consists of two domains, a GTPase domain (G domain) common to members of the GTPase superfamily, and an α-helical domain unique to the highly homologous family of the heterotrimeric G-proteins. It has been reported that the domains of $G_s\alpha$ that are required for adenylate cyclase activation are three regions within the G domain (the C-terminus of the α2 helix, the loop connecting helix α3 to strand β5, and lastly the loop that connects helix α4 to strand β6) [24,25]. Furthermore, the helical domain, probably the B/C loop, is involved in $G_s\alpha$ coupling with adenylate cyclase [26]. There are also roles postulated for the α-helical domain in terms of increasing the affinity of GTP binding and stabilizing the G domain from thermal inactivation [27]. During the present experiments, calpain cleaved $G_s\alpha$, presumably at linker 1, and generated the C-terminal 39-kDa fragment with the deletion of approx. 13 kDa of the N-terminal portion, which, however, contained the B/C loop of the α-helical domain and the complete G domain of $G_s\alpha$. It seems likely that the calpain action on adenylate cyclase activation is due to the truncation of $G_s\alpha$ to the 39-kDa fragment, and this fragment is potentially more active for adenylate cyclase activation than native 52 kDa, because of the loss of the $\beta\gamma$-targeting sequence. These ideas are consistent with the conclusion that mutation at the N-terminus of $G_s\alpha$ releases the attenuator that controls the G_s protein (probably via interaction with $\beta\gamma$), and results in a dominant active G-protein [28]. On the other hand, calpain attacked another cleavage site, possibly at linker 2, and generated a C-terminal 20-kDa fragment, which contained only a part of the G domain. It seems that the 20-kDa fragment cannot activate adenylate cyclase. The present results, however, do not exclude the possible involvement of other substrates for calpain, such as a truncated form of RGS3 which has inhibitory effects on G_s-mediated signalling pathways [29]. Our finding of proteolytic modification of $G_s\alpha$ by calpain is similar to the recent data; the truncation of PLC-β3 that is mediated by μ-calpain induces a much higher activation by the $\beta\gamma$ subunits [30]. Furthermore, calpain cleavage of protein tyrosine phosphatase 1B results in the generation of an enzymically active form of the phosphatase [31].

Enzyme inhibitors can often provide powerful tools to elucidate the functional significance of the enzyme in question, provided that the inhibitors are relatively selective. Here, we have showed that ZLLal, which is a strong inhibitor of calpain but a poor one of proteasomes [18], inhibited the down-regulation of $G_s\alpha$ and sustained cAMP accumulation by cholera toxin or PACAP. On the other hand, an inhibitor that preferentially inhibits proteasome activity, ZLLLal, did not inhibit the cAMP production by cholera toxin (K. Sato, Y. Yajima and S. Kawashima, unpublished work). These results suggest that calpain is involved in the cholera toxin- or PACAP-mediated actions in the GH_4C_1 cells. It has been reported that many distinct fragments are generated from proteins such as PLC-β3 [8], $pp60^{src}$ [9] or focal adhesion kinase $pp125^{FAK}$ [10] upon calpain activation in platelets. In contrast, we have not observed fragments of $G_s\alpha$ protein in cholera toxin- or PACAP-treated cells, which are results similar to those found with $G_q\alpha/G_{11}\alpha$ [3], PKCϵ [19] and cyclin D_1 [32]. At present, we cannot account for the reasons underlying this. Nevertheless, it is still curious that cholera toxin has been shown, in several cell lines, to lead routinely to a permanent stimulation of adenylate cyclase, despite dramatically altering steady-state levels of $G_s\alpha$, since it has been suggested that the amount of available G_s is rate-limiting for the stimulation of cAMP synthesis, as in pseudohypoparathyroidism [33]. Our findings, however, conclusively show that the truncated 39-kDa fragment induced a sustained activity of adenylate cyclase in cholera toxin-treated membranes, and it is more reasonable than the explanation offered by Chang and Bourne [5] that the concentration of $G_s\alpha$ in the GH_3-cell membranes is substantially higher than that required for maximal activation of adenylate cyclase. In conclusion, calpain could be involved in $G_s\alpha$ activation by a new mechanism of signal transduction in rat pituitary cells.

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