Multi-site phosphorylation of the inhibitory guanine nucleotide regulatory protein G_i-2 occurs in intact rat hepatocytes

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A phosphorylated form of α -G_i-2 (the α -subunit of G_i-2), immunoprecipitated from hepatocytes under basal conditions, migrated as a single species of pI $\sim$ 5.7, the labelling of which increased $\sim$ 2-fold in cells challenged with either vasopressin or phorbol 12-myristate 13-acetate (PMA); agents which activate protein kinase C. In contrast, treatment of hepatocytes with 8-bromo-cyclic AMP produced a more acidic species of phosphorylated α -G_i-2 having a pI of $\sim$ 5.4 and whose labelling was increased $\sim$ 3-fold. Trypsin digestion of labelled α -G_i-2 isolated from hepatocytes under basal conditions identified, on two-dimensional peptide analyses, three positively charged phosphoserine-containing peptides (C1, C2 and C3), with only peptides C1 and C2 being evident upon less extensive digestion with trypsin. These are suggested to reflect a single site of phosphorylation, with proteolysis by trypsin being incomplete, and where C2 is larger than C1, which is larger than C3. An

identical pattern of tryptic phosphopeptides was seen in hepatocytes treated with either vasopressin or PMA, although labelling of this group of peptides was increased by $\sim$ 2-fold compared with the basal state. In contrast, treatment of hepatocytes with glucagon, 8-bromo-cyclic AMP or forskolin not only resulted in increased labelling of the 'basal' sites $\sim$ 3-fold, but identified a novel positively charged tryptic phosphoserine-containing peptide (AN). All four tryptic peptides were susceptible to proteolysis by V8 protease. Treatment of labelled α -G_i-2 from basal and PMA-treated cells produced a pattern of peptides which was identical with those found when the tryptic phosphopeptide was treated with V8 protease. We tentatively suggest that, on α -G_i-2, Ser¹⁴⁴ is phosphorylated through the action of protein kinase C and Ser²⁰⁷ is phosphorylated upon elevation of the intracellular concentrations of cyclic AMP.

INTRODUCTION

Many cell-surface receptors control the activity of their effector signal-generating systems through specific members of a family of G-proteins (Birnbaumer et al., 1990). The receptor-dependent production of cyclic AMP (cAMP) is effected by adenylate cyclases whose activities are regulated by two distinct heterotrimeric G-proteins termed, generically, G_s (stimulatory) and G_i (inhibitory). These G-proteins are characterized by unique α -subunits which are the products of different genes. A family of 'G_i-like' proteins has been identified which comprises three highly related pertussis-toxin-sensitive G-proteins termed G_i-1, G_i-2 and G_i-3 (Birnbaumer et al., 1990). Although the specificity of these 'G_i-like' proteins is not yet fully characterized, a number of groups have provided independent evidence that G_i-2 can act as an inhibitory ('G_i') G-protein controlling adenylate cyclase activity (Simonds et al., 1989; Senogles et al., 1990; McKenzie and Milligan, 1990; Bushfield et al., 1990a; Remaury et al., 1993).

In various cell types, the activation of protein kinase C (PKC) has been shown to result in alterations in the regulation of adenylate cyclase activity (reviewed by Houslay, 1991a). In some systems enhanced basal and agonist-stimulated actions have been observed, whereas, in others, a loss of receptor-mediated stimulation was recorded. The underlying molecular basis for these differences may lie in the cell-specific expression of particular control systems, where changes may be dependent on the

expression of particular isoforms of PKC, adenylate cyclase and cAMP phosphodiesterase, as well as the susceptibility of particular receptors to be phosphorylated. However, evidence from a number of studies has indicated that the inhibitory regulation of adenylate cyclase, mediated through G_i, can be prevented by the action of PKC (see Houslay, 1991a; Bushfield et al., 1991). In this regard, we (Pyne et al., 1989; Bushfield et al., 1990a, 1991) and others (Rothenberg and Kahn, 1988; Daniel-Issakani et al., 1989) have shown that PKC activation can lead to the phosphorylation of the α -subunit of the G_i-2 isoform (α -G_i-2) and to the loss of GTP-elicited G_i functioning in hepatocytes, U-973 cells and platelets. Such phosphorylation, however, only appears to occur in certain cell types (Houslay, 1991a), and an additional mechanism for the PKC-mediated loss of G_i inhibition needs to be identified. This may be due to the phosphorylation of the catalytic unit of adenylate cyclase (Chen and Iyengar, 1993). However, even this mechanism may itself be cell-specific, as such an action is apparently restricted to the type-II isoform of adenylate cyclase (Chen and Iyengar, 1993).

In intact hepatocytes, treatment with a range of ligands capable of activating protein kinase C, including phorbol 12-myristate 13-acetate (PMA), vasopressin and angiotensin II, resulted in the selective serine-specific phosphorylation of α -G_i-2, but did not affect the labelling of either α -G_i-3 or α -G_s (Bushfield et al., 1990a). Furthermore, exposure of hepatocytes to the phosphoprotein phosphatase (1 and 2A) inhibitor okadaic acid also caused the phosphorylation of G_i-2 (Bushfield et al., 1991). On

Abbreviations used: G_i, inhibitory G-protein controlling adenylate cyclase activity; G_s, stimulatory G-protein controlling adenylate cyclase activity; PMA, phorbol 12-myristate 13-acetate ('TPA'); TPCK, tosylphenylalanylchloromethane; PKA, protein kinase A; PKC, protein kinase C; cAMP, cyclic AMP; DNP-, dinitrophenyl.

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this basis we have suggested (Bushfield et al., 1991; Houslay, 1991b, 1993) that the activity of this key G-protein may be controlled, in hepatocytes, by a phosphorylation/dephosphorylation cycle. In addition, however, ligands capable of activating cAMP-dependent protein kinase (protein kinase A; PKA), such as glucagon and 8-bromo-cAMP, caused further phosphorylation (Bushfield et al., 1990a), which suggested to us that this G-protein may be susceptible to multi-site phosphorylation. Here we have used two-dimensional peptide-mapping techniques to address this issue.

EXPERIMENTAL

Materials

[32 P]P_i was obtained from Amersham International. PMA was from Cambridge Bioscience. Hormones and Protein A-agarose were from Sigma. Cellulose t.l.c. plates were from Eastman Kodak Co., Rochester, NY, U.S.A. Tosylphenylalanylchloromethane (TPCK)-treated trypsin, α -chymotrypsin and *Staphylococcus aureus* V8 protease were obtained from Lorne Laboratories, Reading, Berks., U.K. Okadaic acid was from Mona Bioproducts, Hawaii, U.S.A. All other biochemicals were from Boehringer, U.K., and all other chemicals were of A.R. grade, from BDH.

Hepatocyte preparation and labelling conditions

Hepatocytes were prepared as previously described (Berry and Friend, 1969; Heyworth and Houslay, 1983) from 220–250 g fed male Sprague–Dawley rats. Cells (10^6 – 10^7 /ml) were preincubated for 50 min at 37 °C in Krebs–Henseleit buffer (50 μ M potassium phosphate, 1 mCi of [32 P]P_i supplemented with 2.5 % (w/v) BSA, 2.5 mM CaCl₂ and 10 mM glucose. Cells were gassed with O₂/CO₂ (19:1) for 30 s every 10 min. Ligands were added in less than 1 % of the total incubation volume and, after an appropriate time, the reactions were stopped by addition of 10 vol. of ice-cold Krebs–Henseleit buffer. The cells were harvested by centrifugation (100 g, 2 min).

Immunoprecipitation of α -G_i-2

This was performed as described previously (Bushfield et al., 1990a). Briefly, a pellet of cells (10^6 /ml) was extracted by the addition of 1 ml of a buffer containing 1 % Triton X-100, 0.1 % SDS, 10 mM EDTA, 100 mM NaH₂PO₄, 100 μ M Na₃VO₄, 2 mM phenylmethanesulphonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 nM okadaic acid and 50 mM Hepes, pH 7.2. After 1 h at 4 °C, non-solubilized material was removed by centrifugation (14000 g; 10 min; 4 °C). Labelled α -G_i-2 was immunoprecipitated by using either SG1 or 1867 antiserum. Both the antisera AS7 and SG1 were raised in rabbits against the C-terminal decapeptide of the α -subunit of transducin (conjugated to keyhole-limpet haemocyanin), and each is capable of recognizing both α -G_i-1 and α -G_i-2, in addition to transducin, but does not recognize either α -G_i-3 or α -G_s (Bushfield et al., 1990a). Antiserum 1867 was raised in rabbits against the C-terminal decapeptide of the α -subunit of G_i-2 (conjugated to keyhole-limpet haemocyanin) and, because of absolute identity over this sequence (see Kaziro, 1990), would be expected to recognize α -G_i-1 as well as α -G_i-2. However, since transducin is expressed specifically in the visual system, and α -G_i-1 cannot be detected in hepatocytes by immunoblotting with specific antisera or by

analysis of mRNA (Griffiths et al., 1990; Bushfield et al., 1990a), then the antisera SG1 and 1867 can both be used as specific tools to immunoprecipitate α -G_i-2 in these cells. Identical results, as regards phosphopeptide profiles, were obtained by using these two antisera in this study, as we had indeed noted previously when using both AS7 and SG1 to analyse hormone- and PMA-induced alterations in the level of α -G_i-2 phosphorylation (Pyne et al., 1989; Bushfield et al., 1990a, 1991). Antiserum (50 μ l) was added to 1 ml of this cell extract and samples were incubated for 12 h at 4 °C. After this period, 50 μ l of Protein A-agarose (25 μ l of packed gel in PBS) was added and the incubation continued for a further 2 h at 4 °C. Immune complexes were collected as Protein A-agarose pellets by centrifugation (4 °C, 100 g, 1 min), and the pellets were washed twice in a buffer containing 1 % Triton X-100, 0.1 % SDS, 100 mM NaCl, 50 mM NaH₂PO₄ and 50 mM Hepes, pH 7.2, and once with the above buffer but lacking SDS. Immunoprecipitation of α -G_i-2, detected by subsequent immunoblotting or the presence of an ~40 kDa phosphorylated species, only occurred in the presence of a specific antiserum in the incubation mixture. It was not apparent if immunoglobulins from pre-immune sera or sera raised against a decapeptide to α -G₂ were used, nor if Protein A-agarose alone was employed. Furthermore, it could be competed out with the C-terminal decapeptide from either α -transducin or α -G_i-2, but not the C-terminal decapeptide from α -G₂ (see Bushfield et al., 1990a, and the present paper). Analyses done with competing peptides were performed as described previously by us (Bushfield et al., 1990a).

Gel electrophoresis and autoradiography

Before SDS/PAGE, Protein A-agarose pellets were resuspended in Laemmli (1970) sample buffer and placed in a boiling-water bath for 3 min. Samples were then centrifuged (14000 g, 2 min) and the supernatants taken for SDS/PAGE. This was performed at 60 mA for 2 h in 10 % acrylamide gels. After electrophoresis, gels were dried and subjected to autoradiography. Gels were scanned and analysed quantitatively by using a Shimadzu C5-9000 dual-wavelength flying-spot scanning densitometer. Labelled bands of interest were excised and radioactivity was determined by Čerenkov counting. Two-dimensional gel electrophoresis of immunoprecipitated α -G_i-2 was carried out as described by O'Farrell (1975), with the pH gradient being determined in each instance by the use of commercial marker proteins (Sigma) of established pI values (namely IEF MIX 3.6–9.3). In addition, pH gradients of two or three control tube gels run in parallel to the duplicate (or triplicate) experiments were also checked by slicing up the gels and determining the pH value of the slices. These served to corroborate findings observed with the marker proteins.

Immunoblotting α -G_i-2 and α -G_i-3 in SG1 immunoprecipitates

Proteins were transferred electrophoretically from polyacrylamide gels on to nitrocellulose, and this was blocked for 2 h with 5 % (w/v) low-fat milk protein in PBS. Primary antiserum (1:200 dilution) in 1 % low-fat milk protein in PBS was then added and incubated for 16 h at room temperature. Blots were then washed extensively in PBS containing 0.2 % (v/v) Nonidet P-40 before incubation for a further 2 h with secondary antiserum, donkey anti-rabbit IgG coupled to horseradish peroxidase (Scottish Antibody Production Unit, Wishaw, Scotland, U.K.), in 1 % (w/v) low-fat milk protein in PBS. The blots were then washed as before in PBS containing 0.2 % Nonidet P-40. The antibody complex was detected by using *o*-dianisidine hydro-

chloride (Sigma) as substrate. As described above, the antisera SG1 and 1867 were used to detect α -G_i-2 and, as described previously (Mitchell et al., 1989), the antiserum I3B was used as specific reagent to detect α -G_i-3.

Elution of ³²P-labelled α -G_i-2 from polyacrylamide gels and the separation of ³²P-labelled enzymic cleavage products

As a routine, the method of Boyle et al. (1991) was used. Polyacrylamide gel chips containing the samples were rehydrated in 50 mM NH₄HCO₃, 0.1% SDS and 0.5% (v/v) 2-mercaptoethanol for 5 min at room temperature before being homogenized. Elution was carried out in two steps, each of 3 h, at 37 °C to give a final volume of 1.2 ml. The eluate was cleared by centrifugation (10 min, 14000 g), and 20 µg/ml carrier protein (BSA) and trichloroacetic acid [final concn. 20%, (w/v)] were added. The samples were then left on ice for 1 h to allow the protein to precipitate. The protein was collected by centrifugation (10 min, 14000 g, 4 °C), washed with ice-cold acetone and allowed to air-dry. The pellets were then dissolved in 50 µl of cold performic acid [98% formic acid/30% H₂O₂ (9:1, v/v), incubated for 1 h at room temperature] and incubated on ice for 60 min before 400 µl of water (4 °C) was added and the sample was freeze-dried. In some instances an alternative method of extraction of material from gel chips was employed (Tavare and Denton, 1988), with similar results. The protein was resuspended in 50 µl of 50 mM NH₄HCO₃ (pH 8) and either 10 µl of trypsin (1 mg/ml) or 10 µl of V8 protease (1 mg/ml) was added. The samples were digested for 8 h at 37 °C before addition of a further 10 µl of protease, and incubation was continued for 16 h. In some instances, where stated, a milder digestion with trypsin was done at a lower temperature (30 °C) for periods of 6 h and 16 h. Again, where stated, in some instances, after treatment with trypsin samples were exposed to chymotrypsin (10 µg for 6 h, then 10 µg for 16 h). After such periods of incubation, 400 µl of water was added to the reaction mixtures and samples were freeze-dried before being resuspended in water and again freeze-dried. This sequence of freeze-drying and resuspension was repeated at least four times in order to remove residual NH₄HCO₃. Before the final freeze-drying, the samples were centrifuged (10 min, 14000 g) and the supernatant was collected for freeze-drying. The material accruing from this final freeze-drying was resuspended in 10 µl of pH 1.9 electrophoresis buffer [formic acid (88%)/acetic acid/deionized water (25:28:897, by vol.)] and applied to 20 cm × 20 cm t.l.c. plates (Eastman Kodak). Peptides were first separated by electrophoresis at pH 1.9 for 2 h at 400 V in the presence of marker dyes ϵ -dinitrophenyl (DNP)-lysine (positive charge), DNP-DL-glutamic acid (neutral charge) and xylene cyanol FF (negative charge) and then by ascending chromatography for 3–4 hours [butanol/pyridine/acetic acid/water (15:10:3:12, by vol.)]. The labelled peptides were identified by autoradiography.

For secondary digests, tryptic phosphopeptides were located by autoradiography and the spots scraped from the t.l.c. plates. The resulting powder was subjected to Čerenkov counting before being resuspended in 300 µl of water and vortex-mixed for 1 min. The suspension was then centrifuged at 14000 g_{av} for 2 min at room temperature. The supernatant was collected and the pellet re-extracted, as above, but with 200 µl of water at least once or until the combined supernatants from these extractions were shown to contain > 80% of the original radioactivity. This extract was then freeze-dried and resuspended in 50 µl of NH₄HCO₃ buffer, pH 8, before treatment with V8 protease and analysis as described above.

Phosphoamino acid analysis of tryptic peptides

Phosphoamino acid analysis was carried out as outlined by Boyle et al. (1991). Briefly, autoradiography of t.l.c. plates served to identify phosphopeptides which were then isolated by excising (scraping) the silica from the plate, with the labelled peptide being subsequently recovered by extraction in water. The efficiency of recovery was ascertained by Čerenkov counting. The samples were then freeze-dried, dissolved in 500 µl of 6 M HCl and transferred to tubes which were then sealed under vacuum. The samples were heated to 110 °C for 90 min, and the resulting solutions were collected and freeze-dried again before being dissolved in pH 1.9 buffer and applied to a 20 cm × 20 cm t.l.c. plate (Eastman Kodak). They were then subjected to electrophoresis at 400 V for 30 min at pH 1.9 in the first dimension, air-dried, rotated through 90° and run at 400 V for 30 min at pH 3.5 (in acetic acid/pyridine/deionized water, 10:1:189, by vol.) in the second dimension. Markers of phosphoserine, phosphothreonine and phosphotyrosine were included with the samples, and these were detected by staining with 0.25% (w/v) ninhydrin in acetone.

Prediction of enzyme cleavage products and mass-charge ratios

The prediction of peptide fragments produced by enzymic cleavage and estimations of their mass-charge ratios were based on the methods outlined in Boyle et al. (1991). All possible peptides produced by the enzymes trypsin (cleaves at arginine and lysine) and V8 protease (cleaves at glutamate and aspartate) were predicted on the basis of the known sequence of rat α -G_i-2 (see Kaziro, 1990). The mass-charge ratio of these was calculated from $m_r = keM^{-\frac{2}{3}}$, where m_r is the mass-charge ratio, k is a constant, e is the charge on the peptide and M is the molecular mass of the peptide (Offord, 1966). The charge of the peptide was calculated from the sum of the charges, at pH 1.9, of its constituent amino acids, where the N-terminal amino acid as well as arginine, histidine and lysine all possess a charge of +1, cysteine a charge of approximately -1 and phosphoserine a charge of -1. In predicting possible serine containing peptides arising from cleavage by, for example, trypsin action, we considered all possible partial cleavage products that may arise, for example, due to the occurrence of multiple arginine and lysine residues, adjacent lysine and arginine residues, adjacent aspartate and glutamate residues, and putative adjacent phosphoserine residues. In α -G_i-2, there are 20 serine residues together with 18 arginine residues and 27 lysine residues (see Kaziro, 1990).

RESULTS AND DISCUSSION

Immunoprecipitation of phosphorylated α -G_i-2 from ³²P-labelled rat hepatocytes

In the experiments described in this study, the antisera SG1 and 1867 were both used to immunoprecipitate α -G_i-2 selectively, with similar results. These antisera were produced against decapeptides representing the C-terminal ends of transducin and α -G_i-2, respectively. The high homology (9 residues out of 10) between these two G-proteins over this region (see Kaziro, 1990) allows for cross-reactivity in terms of both immunoblotting and immunoprecipitation, as shown with the antiserum AS7 (see Milligan, 1990), made against a transducin C-terminal decapeptide, and which has also been used by us (Pyne et al., 1989) and others (Rothenberg and Kahn, 1988) to immunoprecipitate phosphorylated α -G_i-2 from hepatocytes. In Figure 1 we demonstrate that a 41 kDa phosphoprotein was immunoprecipitated from ³²P-labelled rat hepatocytes by both of these antisera. This phosphopeptide co-migrated with immunoreactive α -G_i-2 pres-

Figure 1 Antisera SG1 and 1867 immunoprecipitate phosphorylated α -G_i-2, but not α -G_i-3, from 32 P-labelled hepatocytes

In (a), hepatocytes were labelled with [32 P]P_i for 65 min and then subjected to detergent extraction, immunoprecipitation with antiserum SG1 and subsequent SDS/PAGE. Tracks 2 and 4 show immunoblots done on the immunoprecipitates with either antiserum I3B (track 2), which recognizes α -G_i-3, or antiserum SG1 (track 4), which recognizes α -G_i-2. Tracks 1 and 3 show the corresponding autoradiographs for the tracks which were immunoblotted with these two antisera. Note the presence of a 41 kDa phosphoprotein which co-migrated with immunoreactive α -G_i-2 and the absence of immunoreactive α -G_i-3 in the material immunoprecipitated by SG1. The diffuse band occurring at around 60 kDa in the immunoblots represents antibody heavy chains in the immunoprecipitate analysed. The I3B immunoblot (track 2) was 'over-exposed', as made obvious from the 'Ig' band, to maximize sensitivity in order to try to detect the presence of trace amounts of α -G_i-3 in the immunoprecipitate; however, none was apparent. Under the same conditions, this serum did recognize α -G_i-3 in hepatocyte membranes which had not been subjected to prior immunoprecipitation before immunoblotting. In (b), antiserum 1867 was used to immunoprecipitate 32 P-labelled α -G_i-2 from hepatocytes. Lane 1 shows labelled material which was immunoprecipitated with antiserum 1867 added alone. In lanes 2 and 3 antiserum 1867 was used in the presence of two different concentrations of the competing C-terminal α -G_i-2 decapeptide at concentrations of 1 and 10 μ g/ml respectively. Lane 4 shows the labelled material that was found if antiserum 1867 was preincubated with the C-terminal decapeptide from α -G_i-3 (10 μ g/ml). Only use of the α -G_i-2 C-terminal decapeptide competes out the immunoprecipitation of the 41 kDa band (tracks 2 and 3), demonstrating therefore that antiserum 1867 specifically immunoprecipitates α -G_i-2. The results shown are typical of experiments performed three times.

ent in the immunoprecipitates (Figure 1a). Furthermore, we could find no evidence for the presence of immunoreactive α -G_i-3 in immunoprecipitates done with SG1 (Figure 1a), indicating that, as with the antiserum AS7 (Bushfield et al., 1990a, 1991),

Figure 2 Two-dimensional PAGE analysis of phosphorylated α -G_i-2

Hepatocytes were labelled with 32 P and then challenged with (a) vehicle, (b) TPMA (10 ng/ml) for 15 min, and (c) 3,8-bromo-cAMP (300 μ M) for 15 min. After harvesting the hepatocytes, they were subjected to detergent extraction, immunoprecipitation with antiserum SG1 and then two-dimensional PAGE and autoradiography as described in the Experimental section. In (d) a mixture of the solubilized immunoprecipitates from the experiments shown in (b) and (c) were analysed together, showing the presence of two distinct spots. The autoradiographs shown are from a typical experiment which was performed three times with similar results.

the antiserum SG1 specifically immunoprecipitated α -G_i-2 and not the only other G_i protein, α -G_i-3, that can be detected in hepatocytes by immunoblotting (Figure 1a) and by transcript analysis (Griffiths et al., 1990). Immunoprecipitation of α -G_i-2 by SG1 could be inhibited by using the C-terminal decapeptide from transducin (results not shown), but not the equivalent peptide from α -G_s, as shown previously by us (Bushfield et al., 1990a). Here we show (Figure 1b) that the immunoprecipitation of a phosphorylated protein of ~41 kDa by antiserum 1867 could be specifically inhibited by using the C-terminal decapeptide from α -G_i-2, but not with the equivalent peptide from α -G_i-3, indicating that this labelled species is indeed α -G_i-2. The other member of the G_i family, α -G_i-1, is apparently not present in hepatocytes as determined by both immunoblotting, using a specific antiserum (Bushfield et al., 1990b), or by the presence of transcripts as determined by using specific oligonucleotide probes that we have developed (Griffiths et al., 1990). We thus believe that the phosphorylated species that we (Pyne et al., 1989; Bushfield et al., 1990a,b, 1991) and others (Rothenberg and Kahn, 1988) have immunoprecipitated from hepatocytes using three different antisera is indeed α -G_i-2.

We observed in this study that the level of phosphorylation of α -G_i-2, as detected by resolution of the immunoprecipitated species on SDS/PAGE, was increased in response to treatment of intact hepatocytes with vasopressin (10 nM, 5 min) (2.38 ± 0.25 -fold), angiotensin II (10 nM, 5 min) (2.35 ± 0.25 -fold), glucagon (10 nM, 5 min) (3.19 ± 0.20 -fold), 8-bromo-cAMP (300 μ M, 15 min) (3.97 ± 0.54 -fold) or the phorbol ester PMA (10 nM, 15 min) (2.03 ± 0.21 -fold) (values shown are fold increases over the labelling seen under control conditions in the absence of any added ligand; means $\pm$ S.D., $n = 6$). Such data were similar to those that we have reported previously (Bushfield et al., 1990a).

Immunoprecipitated 32 P-labelled α -G_i-2 from untreated cells migrated as a single spot on two-dimensional gel electrophoresis, with a pI of 5.71 ± 0.08 ($n = 3$; Figure 2). This value was identical with that previously reported for phosphorylated α -G_i-2 from

Figure 3 α -G_i-2 tryptic (lower-temperature digestion) phosphopeptides from agonist-stimulated hepatocytes resolved by two-dimensional thin-layer analysis

Hepatocytes were labelled with ^{32}P and then challenged with: vehicle (a); 10 ng/ml PMA, 15 min (b); 10 nM vasopressin, 5 min (c); 1 nM glucagon, 5 min (d); 1 μM glucagon, 5 min (e); 300 μM 8-bromo-cAMP, 15 min (f). After harvesting the hepatocytes, they were subjected to detergent extraction, immunoprecipitation with antiserum SG1, SDS/PAGE and identification by autoradiography. The phosphorylated α -G_i-2 was electro-eluted and then digested with TPCCK-treated trypsin as described in the Experimental section for the 'mild' procedure. ^{32}P -labelled tryptic phosphopeptides were then separated on thin-layer cellulose plates by electrophoresis at pH 1.9 and ascending chromatography, with DNP-lysine as an internal marker dye as described in Experimental section. The autoradiographs shown are from typical experiments, and each condition was performed at least three times with similar results.

U-937 cells (Daniel-Issakani et al., 1989). Treatment of cells with PMA indicated the labelling of this species (1.95 ± 0.05 -fold; $n = 3$), but did not change the pI (5.72 ± 0.07 , $n = 3$; Figure 2). However, 8-bromo-cAMP treatment, which caused a further increase in labelling of α -G_i-2 (3.75 ± 0.33 -fold, $n = 3$; Figure 2), induced a shift in the mobility of α -G_i-2 which was consistent with the production of a more acidic form, with pI of $\sim 5.38 \pm 0.07$ ($n = 3$; Figure 2).

α -G_i-2 is partially phosphorylated in resting hepatocytes (Rothenberg and Kahn, 1988; Pyne et al., 1989; Bushfield et al., 1990a,b, 1991; the present work) and thus the increased labelling of α -G_i-2 seen upon treating cells with PMA, in the absence of any change in pI, suggests that the activation of PKC by PMA serves to phosphorylate unmodified α -G_i-2 rather than to modify already labelled G-protein further. This would be consistent with phorbol ester treatment allowing levels of incorporation to attain ~ 1 mol of ^{32}P /mol of α -G_i-2 (Bushfield et al., 1990a, 1991). Such a conclusion would also be warranted from our observation (Bushfield et al., 1991) that treatment of hepatocytes with the protein phosphatase inhibitor okadaic acid rapidly increased the labelling of α -G_i-2, which attained a stoichiometry of near unity and became insensitive to any action of PMA, implying the

active action of PKC and a phosphatase under basal conditions. The inactivation of the latter, by okadaic acid, leading to the increased labelling.

In contrast with this, treatment of hepatocytes with 8-bromo-cAMP, which elicits the activation of PKA, caused a decrease in the pI of α -G_i-2 (Figure 2). This occurred concomitantly with a marked increase in the labelling of α -G_i-2 over and above that seen with PMA (Bushfield et al., 1990a, 1991; see above). Such a change in the pI for immunoprecipitated α -G_i-2 is consistent with the labelling of an additional site on this protein, as suggested previously by us to occur on the basis of dose-dependency studies done with glucagon (Bushfield et al., 1990a) and with phosphorylation studies done using mixtures of ligands (Pyne et al., 1989). As the major fraction of the phosphorylated α -G_i-2 migrates as a pI ~ 5.4 component after treatment with 8-bromo-cAMP, it would seem that most of the α -G_i-2 was phosphorylated at this putative additional site after treatment of cells with this ligand. This would be consistent with our previous studies (Bushfield et al., 1990b, 1991) showing that treatment of intact hepatocytes either with the phorbol ester PMA or with vasopressin yielded a stoichiometry of up to ~ 1 mol of ^{32}P /mol of α -G_i-2, whereas treatment of hepatocytes with glucagon or

Figure 4 α -G-2 tryptic (higher-temperature digestion) phosphopeptides from agonist-stimulated hepatocytes resolved by two-dimensional thin-layer analysis and the action of V8 protease

Hepatocytes were labelled with ^{32}P and then challenged with 10 ng/ml PMA for 15 min (**a, d**) or 1 μM glucagon for 5 min (**b, e**). After harvesting the hepatocytes, they were subjected to detergent extraction, immunoprecipitation with antiserum SG1 or 1867, and SDS/PAGE, and phosphorylated α -G-2 was extracted. In (**a**) and (**b**) samples were digested with TPCK-treated trypsin only under the 'higher-temperature conditions' described in the Experimental section. A schematic of the various phosphopeptides produced is shown in (**c**). The AN phosphopeptide was found uniquely with glucagon challenge at the higher concentration of 1 μM (**b**), but not at 1 nM (results not shown, but see Figure 3d). In (**d**) and (**e**) trypsin digestion was followed by digestion with V8 protease as described in the Experimental section. The AN peptide and all the C peptides were susceptible to proteolysis by V8. The schematic (**f**) shows the novel peptides produced, namely C2', C2'',

8-bromo-cAMP causes the level of labelling to rise to up to ~ 2 mol of ^{32}P /mol of α -G_i-2.

Two-dimensional thin-layer analysis of α -G_i-2 tryptic peptides

To gauge whether α -G_i-2 was subjected to multi-site phosphorylation, we tried to identify ^{32}P -labelled phosphopeptides by two-dimensional mapping analyses. Trypsin digestion of α -G_i-2, done at 30 °C, on resting (basal-state) hepatocytes led to the resolution of two closely migrating phosphopeptides (C1, C2), which were resolved by chromatography, but not by electrophoresis (Figure 3a). The ratio of these two peptides varied between experiments and was influenced by alterations in the time of digestion with trypsin (results not shown), indicating that they reflected a single site of phosphorylation, but with partial digestion of α -G_i-2 with trypsin. Similar examination of α -G_i-2 from hepatocytes that had been treated with PMA, in order to activate PKC, identified an identical pattern of phosphopeptides (Figure 3b), albeit with increasing labelling. The variability in the ratio of the C1 and C2 peptides precluded any meaningful analysis of the PMA-induced increase in labelling of these individual peptides. However, if the total labelling of the C1 and C2 peptides was determined, then an increase of 2.2 ± 0.3 -fold was noted ($n = 3$ different cell preparations). Such data are consistent with the formulation made above, namely that PMA treatment caused the phosphorylation of native α -G_i-2 at the same site where a fraction of this G-protein was phosphorylated under basal conditions. This also concurs with our previous stoichiometry studies (Bushfield et al., 1991), which implied a single site for the phosphorylation of α -G_i-2 by PKC.

As well as using the tumour-promoting phorbol ester PMA to activate PKC, we also investigated the effect of the hormone vasopressin, which stimulates phosphoinositide hydrolysis and diacylglycerol production in hepatocytes (Kirk et al., 1981) and activates protein kinase C (Tang and Houslay, 1992). Vasopressin increased labelling of the C-group of peptides (1.8 ± 0.2 -fold; $n = 3$), with no evidence for the appearance of any other labelled species (Figure 3c). This supports our suggestion (Bushfield et al., 1991) that activation of PKC, either directly by PMA or as a result of vasopressin-induced diacylglycerol production, results in the phosphorylation of unmodified α -G_i-2 at the same time as that found to be phosphorylated in a small fraction of α -G_i-2 in resting cells.

There is considerable evidence to support the notion that glucagon can exert actions in hepatocytes which are independent of cAMP (for reviews, see Houslay, 1991a, 1993). This is undoubtedly attributable to the fact that glucagon can activate PKC (Pittner and Fain, 1991; Tang and Houslay, 1992) and can also increase intracellular free $[\text{Ca}^{2+}]$ in two phases, one of which is cAMP-independent (Mine et al., 1988). The mechanism through which these effects occur may involve the very small stimulation of phosphoinositide metabolism that has been observed by us and others (Wakelam et al., 1986; Blackmore and Exton, 1986; Williamson et al., 1986; Whipps et al., 1987), and a robust stimulation of phosphatidylcholine metabolism (Pittner and Fain, 1991), which leads to the noted glucagon-stimulated production of diacylglycerol in hepatocytes (Bocckino et al.,

1985; Pittner and Fain, 1991). Such actions of glucagon appear, however, to occur at lower glucagon concentrations than those required to activate adenylate cyclase. The stimulation of multiple signalling pathways by glucagon may then result from a single receptor able to stimulate at least two G-proteins, one of which is coupled to adenylate cyclase and the other to a phospholipase. Alternatively, multiple splice variants of the receptor may activate different G-proteins selectively, as has been noted for other members of the G-protein-linked receptor sub-family to which the glucagon receptor appears to belong (Jelinek et al., 1993; Spengler et al., 1993). We have shown previously (Bushfield et al., 1990a) that the glucagon-stimulated phosphorylation of α -G_i-2 is biphasic, with low concentrations of glucagon (~ 1 nM) causing a cAMP-independent phosphorylation which parallels loss of GTP-driven G_i inhibition, as is also seen with treatment of cells with either vasopressin or PMA. Here we see (Figure 3d) that challenge of hepatocytes with 1 nM glucagon increased labelling of the C-group of peptides (2.1 ± 0.2 -fold; $n = 3$) in a fashion identical with that found with both PMA and vasopressin. This is consistent with glucagon, at such concentrations, being able to activate PKC in hepatocytes (Tang and Houslay, 1992). In contrast with this, using higher concentrations of glucagon (1 μM), where maximal phosphorylation of α -G_i-2 and production of cAMP occurs (Bushfield et al., 1990a), we then see the appearance of a novel phosphopeptide 'AN', having distinct chromatographic and electrophoretic properties (Figure 3e). Under such conditions, the increase in labelling of the C-peptide group, over that seen under basal conditions, was identical with that found with low concentrations of glucagon (2.0 ± 0.3 -fold; $n = 3$), indicating that all of the increased labelling of α -G_i-2, occurring at high concentrations of glucagon, was at the new site represented by the 'AN' peptide. The stoichiometry of labelling of α -G_i-2 at high glucagon concentrations has been shown to be ~ 2 mol of ^{32}P /mol of α -G_i-2 (Bushfield et al., 1990a, 1991), again consistent with there being two sites for phosphorylation on α -G_i-2: one for PKC and one resulting from elevation of $[\text{cAMP}]$. Indeed, the ability of glucagon to increase intracellular free $[\text{Ca}^{2+}]$ has two components, a cAMP-independent one noted at low hormone levels and a cAMP-dependent one seen at higher glucagon concentrations (Mauger et al., 1985; Mine et al., 1988). Furthermore, 8-bromo-cAMP can also increase hepatocyte intracellular free $[\text{Ca}^{2+}]$ (Staddon and Hansford, 1989), and thus can activate hepatocyte PKC (Tang and Houslay, 1992) directly (Ashendel, 1985). As with glucagon, challenge of cells with 8-bromo-cAMP caused the production of the novel 'AN' peptide (Figure 3f), as well as increasing levels of labelling of the C-peptides (2.3 ± 0.2 -fold; $n = 3$) to an extent which was similar to that seen with PMA. Such data show that α -G_i-2 is subject to multi-site phosphorylation in intact hepatocytes and that this can be achieved by glucagon in a biphasic fashion. It also demonstrates that the site which is phosphorylated as a result of an increase in intracellular $[\text{cAMP}]$ is distinct from that which is partially labelled under basal conditions, and it thus represents a novel site of modification.

To explore the possibility that C1 and C2 were partial digestion products, we tried changing the digestion conditions. In doing so, we found that if digestion was performed at 37 °C then a

C3' and AN'. In (g) are shown data for hepatocytes treated with PMA, as above, with subsequent treatment of the immunoprecipitate with V8 alone. Secondary digests, with V8 protease, are shown for the individually isolated and treated tryptic phosphopeptides C1 (h), C2 (i), C3 (j) and AN (k). All of the ^{32}P -labelled tryptic phosphopeptides were separated on thin-layer cellulose plates by electrophoresis at pH 1.9 and ascending chromatography. The final position of DNP-lysine is marked on each plate, 'SF' represents the position of the solvent front, 'Origin' is the point of application of the sample, (+) and (−) indicate the orientation of the electric field, and the bar at the top of the plate indicates the separation of the negative, neutral and positive markers. The autoradiographs show the results from typical experiments, where vehicle and PMA produced spots C1, C2, and C3 (also C2', C2'' and C3' on V8 digestion), and 8-bromo-cAMP, glucagon and forskolin stimulation produced spots C1, C2, C3 and AN upon trypsin digestion and peptides C2', C2'', C3' and AN' after V8. Peptides C1 and AN showed most resistance to V8 digestion. Each condition was performed at least six times with similar results.

further positively charged tryptic phosphopeptide (C3) could be resolved by electrophoresis at pH 1.9, using α -G_i-2 immunoprecipitated from both basal (results not shown) and PMA-treated cells (Figure 4a; schematic Figure 4c). These three peptides were consistently identified by using cell preparations from 12 different animals. As with digestion at 30 °C, in the range of experiments done, there was considerable variation in the ratio of these three peptides, which militated against any useful comparison of changes in their individual labelling occurring on PMA treatment of the cells. However, analysis of total labelling of the C-phosphopeptide pool showed an increase of 2.0 ± 0.3 -fold ($n = 12$ different cell preparations). This suggests that C1 and C2 are capable of being further processed by trypsin, and support the contention that they represent a single phosphorylation site. When a similar digestion strategy was used, performed on cells which had been treated with 1 μ M glucagon, the 'AN' peptide was evident, as was the C3 peptide (Figure 4b; schematic Figure 4c). However, no other peptide was evident which might have signified further processing of the 'AN' peptide. Identical results (not shown) were obtained for cells treated with either 8-bromo-cAMP or the adenylate cyclase activator forskolin (100 μ M) (Birnbaumer et al., 1990).

All three of the 'C'-group peptides (C1, C2 and C3) were shown to be susceptible to cleavage by V8 protease (Figure 4d; schematic Figure 4f). In one series of experiments, immunoprecipitates were digested first with trypsin at 37 °C and directly digested with V8 protease (Figures 4d and 4e) before being resolved by two-dimensional chromatography. In the second instance, immunoprecipitates were digested with trypsin, phosphopeptides were resolved by two-dimensional chromatography, and individual phosphopeptides eluted for subsequent cleavage with V8 protease (Figures 4h-j). These experiments showed that all of the C-peptides produced small amounts of C3', but in addition C1 also produced the phosphopeptide C2', and C2 produced both C2' and C2''. All such peptides were positively charged under the resolution conditions. Such analyses with V8 protease confirm the relatedness of these peptides to each other, indicating that they are partial digestion products. C1 and C2 are thus clearly related, but the fact that C2 produced C2'' indicates that it is larger than C1 and contains a further site for action of V8 protease. That C3 produced only C3' is consistent with C3 being the smallest of the peptides, resulting from the most complete digestion of α -G_i-2 that we were able to achieve. Digestion of phosphorylated α -G_i-2 with V8 protease alone produced peptides corresponding to the peptides C2', C2'' and C3' (Figure 4g). Thus C1, C2 and C3 must all contain V8 cleavage points on either side of the phosphorylation site. The 'AN' peptide was also subjected to V8-protease cleavage, producing the peptide AN' (Figure 4e and 4k; schematic Figure 4g), although increased incubation times of up to 48 h were required to achieve this. With all of such peptides, phosphoamino acid analyses showed that the labelled amino acid was serine (Figure 5).

As the amino acid sequence of rat α -G_i-2 is known (Itoh et al., 1986), this has allowed us to attempt to predict the peptides that might be produced as a result of protease cleavage (see Boyle et al., 1991). Although trypsin will cleave at the C-terminal side of both arginine and lysine, it can also produce partial digestion products as a result of the occurrence of adjacent arginine and lysine, proximity to acidic residues and also to proline, as well as the occurrence of phosphorylated residues near to the point of cleavage (see Boyle et al., 1991; Sorenson et al., 1991; Breddam and Meldal, 1992). Such occurrences are common in α -G_i-2 (Itoh et al., 1986, 1988), which, together with the fact that there are 20 serine residues in this protein, 18 arginine residues and 27 lysine

Figure 5 Phosphoamino acid analysis of tryptic peptides C1, C2, C3 and AN

Peptides C1, C2, C3 and AN were recovered from t.l.c. plates and subjected to phosphoamino acid analysis as outlined in the Experimental section. Peptide C1 was applied to position 1, C2 to position 2, C3 to position 3 and AN to position 4. The plate was run at pH 1.9 in the first dimension and rotated anticlockwise through 90° before being run in the second dimension. P_i represents phosphate liberated from the peptide as a result of hydrolysis. P-Ser, P-Thr, and P-Tyr represent the final position of the applied standards phosphoserine, phosphothreonine and phosphotyrosine. All the peptides produced phosphoserine, therefore confirming that the phosphorylation sites are serines.

residues, severely complicates analyses. Furthermore, features of secondary structure can also affect susceptibility to proteolysis, as has been ably demonstrated for the related G-protein transducin (Hingorami and Ho, 1990). In contrast with trypsin, V8-protease cleavage occurs predominantly at glutamate residues, but can also occur at aspartate residues with actions affected by nearby positively charged groups and changes in secondary structure (see Breddam and Meldal, 1992; Sorensen et al., 1991). Indeed, we experienced this when α -G_i-2 became resistant to digestion with V8 protease after isolation from cells treated with 8-bromo-cAMP.

Our data indicate the presence of a single phosphorylation site for protein kinase C within α -G_i-2 where various partial digestion products were formed upon trypsin cleavage. We have attempted to analyse such data using two strategies. Firstly, we determined the charge of all possible serine-containing peptides that might be produced through the action of trypsin and V8, so as to assess their relative mass-charge ratios and the changes that would occur upon V8-protease cleavage of tryptic phosphopeptides. Secondly, we considered whether particular serine residues might fall within a motif which would make them potential substrates for phosphorylation by PKC. Although there is some diversity as regards such motifs (Kennelly and Krebs, 1991; Pearson and Kemp, 1991; Azzi et al., 1992; Hug and Sarre, 1993), we have identified seven potential sites in α -G_i-2, namely Ser¹⁶, Ser⁴⁴, Ser⁴⁷, Ser¹⁴⁴, Ser²⁰⁷, Ser²⁴⁷ and Ser³⁰⁶. These are identifiable as: AERS¹⁶KMID; GES⁴⁴GKS⁴⁷TIV; FGRS¹⁴⁴REY; GQRS²⁰⁷ERK; RMHES²⁴⁷MKL; YIQS³⁰⁶KFED.

Potential candidates would be those serines which contained sites for V8-protease cleavage on either side and which were contained within tryptic peptides able to produce V8 peptides of elevated mass-charge ratio. Such parent tryptic phosphopeptides would also have to produce sufficient V8 peptides to account for the occurrence of C2', C2'' and C3'. On this basis, if the putative parent tryptic peptide was deduced to produce a V8 peptide product with zero or a net negative charge, then these peptides, and their parent tryptic peptides, were eliminated, as we noted that all the V8 phosphopeptides produced were positively charged (Figures 4d–4g). This approach allowed us to eliminate all serine residues except for Ser⁴⁷ and Ser¹⁴⁴, with only Ser¹⁴⁴ generating sets of V8 peptides with appropriate relative mass-charge ratios. Such data indicates that Ser¹⁴⁴ is the site for phosphorylation by PKC on α -G_i-2. This residue is also proposed to have a surface localization, where it should be available for PKC to modify, based on predictions concerning the secondary structure of α -G_i-2 (Kaziro, 1990; Conklin and Bourne, 1993) and is believed to lie within a domain involved in coupling to the effector, adenylate cyclase.

Although elevations in intracellular [cAMP] elicit phosphorylation of α -G_i-2 at a second site, it is not known whether or not this results from the direct action of PKA, as there is evidence which suggests that, when isolated membranes are used, G_i-2 is not phosphorylated by purified PKA (see Houslay, 1991a). Thus one cannot make any *a priori* decisions on the possible motif surrounding the target serine in AN. We have therefore considered all potential serine-containing tryptic peptides in order to identify species where, after cleavage with V8, a peptide would be produced whose mass-charge ratio was decreased, as observed in the production of AN' from AN. Such an analysis points only to Ser²⁰⁷ as the target residue. Indeed, this residue has an arginine group to the N-terminal side, which, of course, might allow it to serve as a substrate for PKA, albeit a weak one. Predictions of secondary structure (Kaziro, 1990; Conklin and Bourne, 1993) would also place this group near the surface of the molecule, but, in this instance, near the GTPase domain.

Using the above strategy to eliminate peptides of inappropriate charge, then, from those remaining, we can produce a tentative scheme for the production of a series of digestion products which are compatible with the data observed. On this basis we suggest that C2, the largest peptide we observed, might have an identity as:

Q¹⁰⁶LFALSCAAEEQGMLPEDLSGVIRR¹³⁰LWADHGVQACFGRS¹⁴⁴REYQLNDSAAYYLNDLERIAQSDYIPTQQD-VLR¹⁷⁸

This could undergo further digestion by trypsin to yield C1, as:

L¹³¹WADHGVQACFGRS¹⁴⁴REYQLNDSAAYYLNDLER¹⁶².IAQSDYIPTQQDVLR¹⁷⁸

and C3 as:

L¹³¹WADHGVQACFGRS¹⁴⁴REYQLNDSAAYYLNDLER¹⁶²

We would suggest that trypsin might be quite resistant to acting at either R¹⁴³ or R¹⁴⁵, due to the phosphoserine which is suggested to be found between these two residues. A similar situation may also hold true for trypsin action at R¹⁶², as this residue has an acidic glutamate on its N-terminal side, thus explaining our observation that it is difficult to achieve full breakdown to C3, and hence the occurrence of partial digestion products. As discussed above, although we routinely observed the formation of C1 and C2, we noted considerable differences in the proportion of these labelled phosphopeptides in various

experiments. Cleavage of C2, in this instance, to give C1 is proposed to occur at R¹³⁰, for which there is an adjacent arginine. We have to presume that the large peptide formed by C2 has sufficient secondary structure such that there is a degree of hindrance to the access of trypsin to this region, yielding partial digests of C1 and C2.

All three peptides, as well as native α -G_i-2, produce C3' upon treatment with V8 protease (Figure 4). This indicates that the phosphorylated serine can, in all instances, be excised by V8 treatment. It is possible to envisage such a scenario with the peptides indicated above, where the sequence of C3' is suggested to be:

H¹³⁵GVQACFGRS¹⁴⁴REYQLND¹⁵¹

We suggest that C3' has the above sequence, rather than terminating at E¹⁴⁶. This is because such a foreshortened peptide would have a considerably higher mass-charge ratio than those for either C1 or C2. Indeed, it is possible that the arginine residue, which is C-terminal to E¹⁴⁶, might attenuate the action of V8 protease at such a site. The other possible site for action of V8 on C3 would be at E¹⁶¹. However, this too lies adjacent to an arginine residue, which might be expected to attenuate its activity. Action at such a residue would also generate a peptide of inappropriate charge, which eliminates it from consideration.

V8 treatment of both C1 and C2 generates a peptide which we have called C2' (Figure 4). We suggest that this might be:

H¹³⁵GVQACFGRS¹⁴⁴REYQLNDSAAYYLNDLERIAQSD¹⁶⁷

The alternative candidate would be for termination at D¹⁵⁹. However, this would give a peptide of inappropriate charge.

C2 also produces a further peptide, C2'', upon treatment with V8 protease (Figure 4). This is a partial digestion product which is unique to C2. Thus the position where V8 cleavage forms the N-terminal end of this peptide would have to occur at one of four possible positions, namely E¹¹⁶, E¹¹⁷, E¹²² or D¹²³. There are thus a large number of possible V8 peptides that may be generated. The strategy that we have adopted can be used to exclude certain of these on the basis of inappropriate charge, leaving the most likely candidate for C2'' as:

L¹²⁴SGVIRRLWADHGVQACFGRS¹⁴⁴REYQLND¹⁵¹

This assumes V8 cleavage after D¹²³, generating an appropriately charged peptide, and also at D¹⁵¹, as in C3', leaving C2'' as a partial digestion product which contains C3'.

We have suggested that Ser²⁰⁷ may provide the site for phosphorylation in the AN peptide. Various tryptic peptides containing this residue can be seen to be nested within a pair of residues where V8 might act. As seen below, tryptic cleavage after K¹⁹⁸ could generate a family of peptides with ambiguity as to the residue at the C-terminus:

M¹⁹⁹FDVGGQRS²⁰⁷ERKK

However, of these only

M¹⁹⁹FDVGGQRS²⁰⁷ERK²¹⁰

would generate a peptide of appropriate charge. Presumably, cleavage after R²⁰⁹ is not favoured, due to the adjacent glutamate residue. We thus suggest that this peptide might reflect the sequence of AN. Treatment with V8 would be expected to cleave at D²⁰¹ and, in order for an appropriately charged peptide to be produced, also at E²⁰⁸ to yield AN', suggested to be:

VGGQRS²⁰⁷E

The assignments made above, as regards the identity of the peptides generated by both V8 cleavage and trypsin cleavage, are

tentative. However, the serines considered here are the only species whose phosphorylation could generate a series of appropriately charged peptides which could account for our observations.

The inhibitory G-protein α -G_i-2 appears to play a pivotal role in controlling the functioning of adenylate cyclase (Birnbaumer et al., 1990; Bushfield et al., 1991). This is achieved both by mediating the action of inhibitory receptors on adenylate cyclase, and also by exerting a constitutive or tonic inhibition due to the high intracellular [GTP] that is found. A phosphorylation/dephosphorylation cycle acting at this site may serve to regulate and/or 'fine-tune' the control of adenylate cyclase functioning through this G-protein (Houslay, 1991b). Here we have shown that α -G_i-2 is clearly subject to multi-site phosphorylation. We tentatively suggest that this occurs through the action of PKC both on Ser¹⁴⁴, and, as a consequence of elevation of intracellular [cAMP], on Ser²⁰⁷. Interestingly, Lounsbury et al. (1993) have shown in studies of G_z, a G-protein of unknown function, that PKC can cause its phosphorylation on Ser²⁷, a residue, cognate to which, is not found in α -G_i-2 (Kaziro, 1990).

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