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Opioid peptides promote cholera-toxin-catalysed ADP-ribosylation of the inhibitory guanine-nucleotide-binding protein (G_i) in membranes of neuroblastoma × glioma hybrid cells

Graeme MILLIGAN*†‡ and Fergus R. McKENZIE*
Molecular Pharmacology Group, Departments of *Biochemistry and †Pharmacology, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

NG108-15 neuroblastoma × glioma hybrid cells express a major 45 kDa substrate for cholera toxin and a 40 kDa substrate(s) for pertussis toxin when ADP-ribosylation is performed in the presence of GTP. In the absence of exogenous GTP, however, cholera toxin was shown to catalyse incorporation of radioactivity into a 40 kDa protein as well as into the 45 kDa polypeptide. In membranes of cells which had been pretreated *in vivo* with pertussis toxin, the 40 kDa band was no longer a substrate for either pertussis or cholera toxin *in vitro*, whereas in membranes from cholera-toxin-pretreated cells the 40 kDa band was still a substrate for fresh cholera toxin *in vitro* and for pertussis toxin. In this cell line, opioid peptides have been shown to inhibit adenylate cyclase exclusively by interacting with G_i (inhibitory G-protein) and with no other pertussis-toxin-sensitive G-protein. Opioid agonists, but not antagonists, promoted the cholera-toxin-catalysed ADP-ribosylation of the 40 kDa polypeptide, hence demonstrating that this cholera-toxin substrate was indeed the α-subunit of G_i. These results demonstrate that G_i can be a substrate for either cholera or pertussis toxin under appropriate conditions.

INTRODUCTION

Exotoxins isolated from cultures of *Vibrio cholerae* and *Bordetella pertussis* have been invaluable tools in the identification of the guanine-nucleotide-binding regulatory proteins (G-proteins) of the adenylate cyclase second-messenger-generation system [1,2]. It is generally assumed that cholera toxin catalyses mono-ADP-ribosylation of the α-subunit of G_s, but not that of G_i. In contrast, pertussis toxin is assumed to ADP-ribosylate G_i, but not G_s. The cysteine residue which is the site of action of pertussis toxin in G_i is not conserved in G_s [3,4]. This hence offers a rationale for the lack of effect of pertussis toxin on G_s. However, this cysteine residue is conserved in a number of other G-proteins, such as G_o and transducin, and these have also been demonstrated to be substrates for pertussis toxin. The arginine residue which is the target for cholera-toxin-catalysed ADP-ribosylation in G_s is conserved in the equivalent position in all G-proteins which have currently been identified by analysis of cDNA clones [3,4]. It thus appears likely that the conformational structure of certain G-proteins, rather than the primary sequence of the amino acid chain, may be responsible for the apparent lack of sensitivity of these proteins to cholera toxin. Here we demonstrate, in membranes of the neuroblastoma × glioma hybrid cell line NG108-15, that the α subunit of one of the non-allelic forms of G_i can indeed function as a substrate for cholera toxin under conditions in which the G-protein does not bind a guanine nucleotide.

δ-Opioid receptors in the plasma membrane of these cells have previously been demonstrated to inhibit adenylate cyclase (see [2], for example) via a pertussis-toxin substrate [5] which has been shown to be a form of G_i [6]. Agonists at the δ-opioid receptor, but not antagonists, stimulated the cholera-toxin-catalysed ADP-ribosylation of G_i, providing a novel and unusual method of demonstrating that in NG108-15 membranes the δ-opioid receptor interacts with a form of G_i. These observations may be generally applicable to studies of interactions of receptors with pertussis-toxin-sensitive G-proteins.

MATERIALS AND METHODS

NG108-15 cells, originally kindly given by Dr. W. Klee, National Institutes of Health, Bethesda, MD, U.S.A., were routinely grown in 80 cm^2 tissue-culture flasks in Dulbecco's Modified Eagle's Medium containing 10% (v/v) foetal bovine serum (Imperial Laboratories) which had been treated at 56 °C for 0.5 h before use. This medium was supplemented with hypoxanthine, aminopterin and thymidine as previously described [7] and both penicillin and streptomycin (100 units/ml). Cells were subcultured after brief trypsin treatment by passaging at a ratio of 1:10. Cells were harvested at confluency and membranes prepared from them after overnight storage at −80 °C [8]. The membranes so produced were stored at −80 °C until use.

Abbreviations used: G_i, inhibitory guanine-nucleotide-binding protein of the adenylate cyclase system; G_i1, G_i2, G_i3, non-allelic variants of 'G_i-like' proteins, identified from cDNA clones and named for the chronology of their discovery (it is unknown whether each of these forms can mediate inhibition of adenylate cyclase or whether they have different functions); G_s, stimulatory G-protein of the adenylate cyclase cascade; G_o, a G-protein of undefined function which is widely distributed, but particularly prevalent in brain; EC_{50}, concentration of ligand required to elicit 50% of the maximal stimulation; DALAMID, [D-alanine²][methionine⁵]enkephalinamide.

‡ To whom correspondence and reprint requests should be addressed.

In some cases cells were treated with either pertussis toxin (Porton Products, Porton Down, Wilts., U.K.) (25 ng/ml) or cholera toxin (Sigma) (100 ng/ml) for 16 h before cell harvest.

ADP-ribosylation of NG108-15 membranes was performed as previously described in either the presence [9] or the absence [10] of GTP. In the relevant ADP-ribosylation assays *in vitro*, pertussis toxin was present at 10 μg/ml and cholera toxin at 50 μg/ml. Incubations were terminated by sodium deoxycholate/trichloroacetic acid precipitation [9] and the samples were resolved by SDS/polyacrylamide (10 %, w/v)-gel electrophoresis at 50 V. Gels were stained with 0.1 % Coomassie Blue, dried and autoradiographed for up to 7 days by using Kodak X-O-Mat X-ray film.

Analysis of the incorporation of radioactivity into polypeptides which were labelled in a toxin-specific manner was performed by scanning the autoradiograms so produced with a Bio-Rad gel scanner linked to an Olivetti M24 personal computer, as previously described by us in some detail [11,12]. Protein was measured by the method of Lowry *et al.* [13].

RESULTS

In the absence of exogenously added guanine nucleotides, cholera toxin catalysed the significant incorporation of radioactivity from [^{32}P]NAD into two polypeptides with apparent molecular masses 45 and 40 kDa in membranes of the neuroblastoma × glioma hybrid cell line NG108-15 (Fig. 1). A less prominent 42 kDa polypeptide was also modified in a cholera-toxin-dependent manner, but, as a further polypeptide which migrated close to the 42 kDa band incorporated radioactivity in a toxin-insensitive fashion, it was difficult to note toxin-dependent effects on the 42 kDa band consistently. Radioactivity was incorporated into certain other polypeptides, but in no other case was this dependent on the presence of toxin in the assay. When a similar experiment was carried out but in the presence of added GTP, then, as noted above, cholera toxin catalysed the incorporation of radioactivity into the 45 and 42 kDa polypeptides, but not into the 40 kDa species (results not shown, but see [2]). The 40 kDa band modified by cholera toxin co-migrated with the only apparent polypeptide to be specifically ADP-ribosylated by pertussis toxin in these membranes (Fig. 1).

After pretreatment of NG108-15 cells with pertussis toxin, cholera toxin in the absence of GTP was able to catalyse ADP-ribosylation of both the 45 and 42 kDa polypeptides, but could no longer modify the 40 kDa species in membranes prepared from these cells (Fig. 2). Pertussis toxin equally was unable to catalyse incorporation of radioactivity into the 40 kDa band *in vitro* after treatment of the cells *in vivo* with pertussis toxin, as we have previously described [6] (results not shown).

In contrast, however, after treatment of the cells *in vivo* with cholera toxin, fresh cholera toxin in the absence of GTP was able to incorporate radioactivity from [^{32}P]-NAD into the 40 kDa polypeptide, but not the 45 and 42 kDa proteins in membranes prepared from these cells (Fig. 2). Pertussis toxin also catalysed ADP-ribosylation of a 40 kDa protein under these conditions (results not shown).

Addition of the synthetic opioid peptide [D-alanine^{2}]-[methionine^{5}]enkephalinamide (DALAMID) enhanced the cholera-toxin-catalysed ADP-ribosylation of the 40 kDa polypeptide, but did not substantially affect the incorporation of radioactivity into either the 45 or 42 kDa bands when the reaction was performed on membranes of untreated control NG108-15 cells in the absence of exogenously added GTP (Fig. 3). Other potent opioid agonists, including [D-alanine^{2}][leucine^{5}]-enkephalin (DADLE) and morphine, produced similar results (results not shown). Over the time course

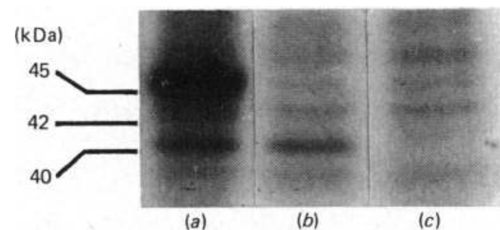

Fig. 1. ADP-ribosylation of membranes of untreated NG108-15 cells by cholera or pertussis toxin in the absence of exogenously added guanine nucleotides

Membranes (30 μg) of these cells were treated with [^{32}P]-NAD and activated cholera toxin (*a*), pertussis toxin (*b*) or without toxin (*c*), in the absence of added guanine nucleotides for 2 h at 37 °C. At the termination of incubation the samples were collected by sodium deoxycholate/trichloroacetic acid precipitation and resolved on an SDS/10 %-polyacrylamide gel. An autoradiogram of the relevant portion of the dried gel is displayed. The only bands labelled in a toxin-dependent manner were of apparent molecular masses 45, 42 and 40 kDa for cholera toxin and 40 kDa for pertussis toxin. To focus attention on these polypeptides, only the relevant area of the autoradiogram is displayed.

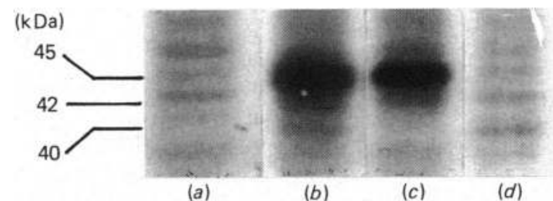

Fig. 2. ADP-ribosylation of NG108-15 membranes by cholera toxin in the absence of exogenously added guanine nucleotides

Membranes were prepared from NG108-15 cells which had been pretreated with either pertussis toxin or cholera toxin or without toxin as described in the Materials and methods section. These membranes were then treated with [^{32}P]NAD and activated cholera toxin as described in the legend to Fig. 1 and separated and autoradiographed as outlined in Fig. 1. (*a*) Labelling of membranes of untreated cells in the absence of toxins [membranes from cells which had been pretreated with either pertussis toxin or cholera toxin displayed the same pattern of labelling as in (*a*)]. (*b*) Cholera-toxin labelling of untreated cell membranes (labelling of 45, 42 and 40 kDa bands; cf. Fig. 1, lane *a*). (*c*) Cholera-toxin labelling of membranes from pertussis-toxin-pretreated cells showed no incorporation of radioactivity into the 40 kDa band. (*d*) Cholera-toxin labelling of membranes from cholera-toxin-pretreated cells showed incorporation of radioactivity into the 40 kDa band, but now showed very little incorporation into the other cholera-toxin substrates

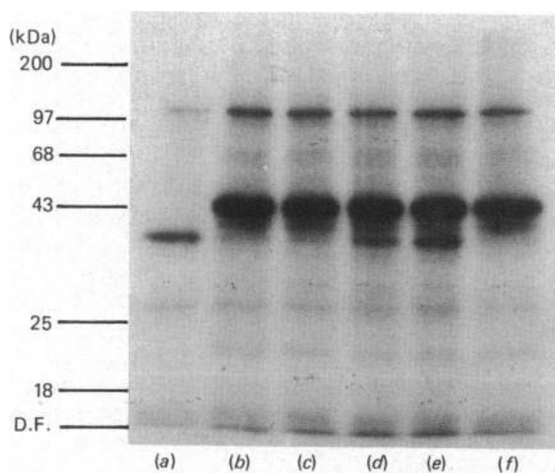

Fig. 3. Pharmacology of the opioid-peptide stimulation of cholera-toxin-catalysed ADP-ribosylation of a 'G_i-like' protein in membranes of untreated NG108-15 cells

Membranes of untreated NG108-15 cells were treated with either pertussis toxin (lane *a*) or cholera toxin (lanes *b–f*) in the absence of guanine nucleotides for 2 h at 37 °C as described in the Materials and methods section. Additionally, lanes (*a*) and (*f*) contained no ligand, lane (*b*) contained naloxone (1 mM), lane (*c*) naloxone (1 mM) + DALAMID (0.1 μM), lane (*d*) DALAMID (0.1 μM) and lane (*e*) DALAMID (10 μM). The samples were collected, resolved and autoradiographed as in Fig. 1; D.F., dye front. Incorporation of radioactivity into the 40 kDa band was also assessed densitometrically as described in the Materials and methods section and in detail in [11]. Taking the incorporation catalysed by pertussis toxin under these conditions (lane *a*) as 100% and incorporation of radioactivity by cholera toxin into this band in the absence of added ligand (lane *f*) as a blank, then lane (*b*) = 4%, lane (*c*) = 7%, lane (*d*) = 41% and lane (*e*) = 66%. Similar data were produced in three separate experiments.

employed in these experiments, 0.1 μM-DALAMID promoted less incorporation of radioactivity into the 40 kDa band than did 10 μM-DALAMID (Fig. 3). This effect was not mimicked by the opioid antagonist naloxone, and the antagonist blocked the DALAMID-mediated effect (Fig. 3; compare lanes *c* and *d*). Dose–response curves of the enhancement of cholera-toxin-catalysed ADP-ribosylation of the 40 kDa polypeptide showed that no effect of DALAMID could be observed at concentrations below 1 nM and that a maximal increase in the cholera-toxin-catalysed ADP-ribosylation of the 40 kDa polypeptide was achieved by 1 μM. The EC_{50} for DALAMID was estimated to be some 16 ± 10 nM (Fig. 4*a*), a value in close agreement with the EC_{50} estimated for DALAMID-mediated stimulation of high-affinity GTPase activity in these membranes [6]. Similar results in terms of both the pharmacological profile of the response (results not shown) and the dose–response curves to DALAMID (Fig. 4*b*) were obtained when membranes of cholera-toxin-pretreated NG108-15 cells were used as the acceptor system for the cholera-toxin-catalysed ADP-ribosylation *in vitro* in the absence of GTP. The cholera-toxin-pretreated system had, however, the distinct advantage that essentially no radioactivity was incorporated into the 45 and 42 kDa bands. Thus the 40 kDa band was the sole polypeptide to be ADP-

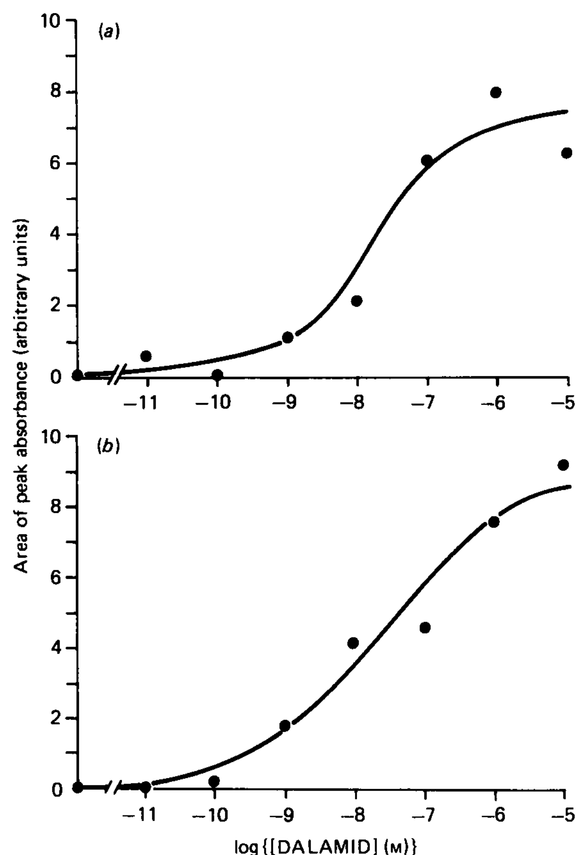

Fig. 4. Dose/response curve of DALAMID stimulation of the cholera-toxin-catalysed ADP-ribosylation of a 'G_i-like' protein in membranes of untreated and cholera-toxin-pretreated NG108-15 cells

(*a*) Cholera-toxin-catalysed ADP-ribosylation of membranes of untreated NG108-15 cells was performed in the absence of guanine nucleotides, as described in the Materials and methods section, in the presence of various concentrations of DALAMID. Densitometric analysis of the 40 kDa band was performed as in Fig. 3, and incorporation of radioactivity into this polypeptide in the absence of ligand was subtracted as a blank. In three separate experiments the estimated EC_{50} for DALAMID was 16 ± 10 nM. (*b*) Cholera-toxin-catalysed ADP-ribosylation of membranes of cholera-toxin-pretreated NG108-15 cells was performed and the products were analysed as described in Fig. 4(*a*).

reibosylated in a cholera-toxin-sensitive manner in membranes from the cholera-toxin-pretreated cells.

DISCUSSION

Until recently it has been tacitly assumed that pertussis-toxin-catalysed ADP-ribosylation provided a specific means of identification of G_i, and cholera-toxin-catalysed ADP-ribosylation was a similar identification of G_s. Particularly with pertussis toxin, this is now recognized to be an oversimplification. Three genes coding for 'G_i-like' proteins have been identified [4], as well as G_o [14–16], all of which are potential substrates for pertussis toxin. All of these proteins contain a conserved arginine residue at a position equivalent to that which is the site for cholera-toxin-catalysed ADP-ribosylation in G_s [3].

Relatively little information has been presented to suggest that these proteins can also act as substrates for cholera toxin; however, it has been noted, in a macrophage cell line [17], neutrophils [18] and the rat glioma cell line C6 BU1 [10], that cholera toxin can catalyse ADP-ribosylation of a 40 kDa protein in assays to which no guanine nucleotide has been added. It has also been noted that cholera toxin can have functional effects on G_i-linked systems in adipocytes [19].

Here we demonstrate definitively that cholera toxin can indeed catalyse ADP-ribosylation of a G-protein which is also a substrate for pertussis toxin, namely the inhibitory G-protein of the adenylate cyclase system, G_i. The evidence presented here in support of this is threefold. (1) Cholera toxin, in the absence but not in the presence of guanine nucleotides, catalysed ADP-ribosylation of a 40 kDa protein which co-migrated with a 40 kDa pertussis-toxin substrate in membranes of the neuroblastoma × glioma hybrid cell line NG108-15. (2) In membranes prepared from NG108-15 cells which had been pretreated *in vivo* with pertussis toxin, before cell harvest, to modify with endogenous NAD, and hence to inactive the pertussis-toxin-sensitive G-proteins present, cholera toxin was unable to catalyse ADP-ribosylation of the 40 kDa protein. However, this toxin was still able to modify the 45 and 42 kDa polypeptides, which represent forms of G_s. (3) Activation of the population of δ-opioid receptors on the surface of these cells with synthetic enkephalin agonists enhanced the cholera-toxin-mediated ADP-ribosylation of the 40 kDa band. This effect was blocked by the opioid antagonist naloxone.

Although a range of pertussis-toxin-sensitive G-proteins, including both G_i and G_o, are expressed in this cell line [20], we have recently demonstrated that the opioid receptor on NG108-15 cells interacts exclusively with a 'G_i-like' protein, by showing that an anti-peptide antiserum which recognizes the *C*-terminal region of the G_i subfamily of pertussis-toxin-sensitive G-proteins, but not of other pertussis-toxin-sensitive G-proteins, such as G_o, uncoupled the opioid receptor from its associated G-protein [6]. Thus the 40 kDa protein which is ADP-ribosylated by pertussis toxin and also by cholera toxin in a manner which is enhanced by opioid peptides must represent the α-subunit of a form of G_i. It remains for definitive immunological examination to identify this protein as G_i1, G_i2 or G_i3 [4]. It is also noteworthy that the EC_{50} for DALAMID promotion of cholera-toxin-catalysed ADP-ribosylation of G_i was very similar to that for DALAMID stimulation of high-affinity GTPase activity, which we have previously reported on in this system [6].

Although pertussis-toxin pretreatment of the cells *in vivo* prevented both pertussis- and cholera-toxin-catalysed ADP-ribosylation of G_i *in vitro*, this effect is not because both toxins have the same target amino acid. As stated above, pertussis toxin catalyses transfer on to a cysteine residue, and cholera toxin that on to an arginine residue. The explanation may lie in the preferred states of the substrate for each toxin. Pertussis-toxin-catalysed ADP-ribosylation of G-proteins is usually decreased in the presence of non-hydrolysable analogues of GTP (see [10], for example), whereas cholera-toxin-catalysed ADP-ribosylation of G_s is enhanced by these analogues [10]. This has been interpreted to imply that pertussis toxin prefers to interact with the holomeric heterotrimeric forms of its substrates and that cholera toxin interacts preferentially with the isolated α-subunit of its substrates. Indeed it has been noted that the isolated α-subunit of G_o is not a substrate for pertussis toxin [21], but becomes so upon addition of β–γ subunit complex. If pertussis-toxin pretreatment of the cells was to stabilize the holomeric form of G_i, then this might represent a poor substrate for cholera toxin. A second possibility is based on the observation that, for pertussis-toxin-sensitive G-proteins to serve as substrates for cholera toxin, no guanine nucleotide should be bound to the protein. Pertussis-toxin-catalysed ADP-ribosylation prevents hydrolysis and release of GTP, and hence this would be expected to prevent cholera-toxin-catalysed ADP-ribosylation of the protein. Further evidence in favour of the requirement for a nucleotide-free form of G_i as a substrate for cholera toxin was previded by the observation that agonist activation of a receptor which has been shown to couple exclusively to G_i promoted the cholera-toxin-mediated ADP-ribosylation of this G-protein. The rate-limiting step in the cycle of G-protein activation and deactivation appears to be the rate of release of GDP (see [22] for a review). The rate of release of GDP is known to be increased by agonist activation [22], and in the absence of exogenously added GTP the GDP will not be replaced, producing a protein stripped of bound nucleotide.

Agonist activation of the cholera-toxin-catalysed ADP-ribosylation of G_i may be a general phenomenon for receptors coupled to G_i and to 'G_i-like' proteins. In this regard we have recently noted that foetal-calf-serum activation of a poorly characterized growth-factor receptor on C6 BU1 cells stimulated cholera-toxin-catalysed ADP-ribosylation of the 'G_i-like' protein G_i2 [23]. It has also been appreciated for some time that transducin, the G-protein of rod outer segments which couples the photon receptor rhodopsin to a cyclic GMP phosphodiesterase, can be a substrate for either cholera or pertussis toxins under appropriate conditions [24]. Experiments similar to those described here may be of general use in defining the interactions of receptors with specific pertussis-toxin-sensitive G-proteins.

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