Volume 269, number 2, 430-434 FEBS 08815 September 1990

The α_2B adrenergic receptor of undifferentiated neuroblastoma × glioma hybrid NG108-15 cells, interacts directly with the guanine nucleotide binding protein, G_i2

Steven J. McClue[1] and Graeme Milligan[1,2]

Molecular Pharmacology Group, Departments of [1]Biochemistry and [2]Pharmacology, University of Glasgow, Glasgow G12 8QQ, Scotland, UK

Received 6 June 1990; revised version received 9 July 1990

In membranes of undifferentiated neuroblastoma × glioma hybrid cell line NG108-15, the apparent specific binding of [^{3}H]yohimbine measured in the presence of 1 μM noradrenaline, was increased substantially by the presence of the poorly hydrolysed analogue of GTP, guanylyl-imidodiphosphate (Gpp[NH]p) or by preincubation of membranes with antibodies against the C-terminal decapeptide of the α subunit of the G-protein G_i2. Such an effect was not produced by antibodies against the equivalent region of $G_o\alpha$ $G_i3\alpha$ or $G_s\alpha$ or from non-immune serum. By contrast, total specific binding of [^{3}H]yohimbine was not modified by co-incubation with Gpp[NH]p or by preincubation with the antibodies from any of the anti-G protein antisera. These results demonstrate a direct interaction of the α_2B adrenergic receptor of NG108-15 cells with G_i2.

α_2B Adrenergic receptor; Guanine nucleotide binding protein; Anti-peptide antiserum; Pertussis toxin

1. INTRODUCTION

The neuroblastoma × glioma hybrid cell line, NG-108-15, is widely used as a model system to study transmembrane signalling cascades [1,2]. These cells express a considerable range of receptors which inhibit adenylate cyclase including those for α2 adrenergic, δ opioid and muscarinic ligands [1]. Receptor-mediated inhibition of adenylate cyclase is transduced via the pertussis toxin-sensitive G-protein 'G_i' [3]. However, the expression of three distinct pertussis toxin-sensitive G-proteins, G_i2, G_i3 and G_o, by NG108-15 cells has been demonstrated both by pertussis toxin-catalysed [^{32}P]ADP-ribosylation [4] and by immunoblotting with specific antipeptide antisera able to discriminate between these G-proteins [4] and hence it is unclear which of these G-proteins functions as 'G_i'.

In this report we assess this question in membranes of undifferentiated NG108-15 cells. We use a series of antisera directed against synthetic peptides which correspond to the C-terminal decapeptides of the α subunits of the individual G-proteins expressed by these cells to attempt to uncouple the α_2B adrenergic receptor from the G-protein signalling system. Uncoupling of receptor and G-protein is known to reduce the affinity of agonists, but not antagonists, for receptors [5]. Thus, when measuring displacement of specific binding of an [^{3}H]antagonist by an agonist, uncoupling and hence a reduction in agonist affinity will result in an increase in specific [^{3}H]antagonist binding. Such effects were observed when either a poorly hydrolysed analogue of GTP, Gpp[NH]p, or an IgG fraction from an antiserum (AS7) able to identify only $G_i2\alpha$ in these cells was used. No such effect was noted with the use of IgG fractions derived from antisera against the equivalent regions of $G_i3\alpha$,$G_o\alpha$ or $G_s\alpha$. These data demonstrate a direct interaction of G_i2 with the α_2B adrenergic receptor in NG108-15 cells and provide a methodology which should be applicable to any receptor which interacts with a G-protein(s).

Correspondence address: G. Milligan, Molecular Pharmacology Group, University of Glasgow, Glasgow G12 8QQ, Scotland, UK

Abbreviations: G-protein, guanine nucleotide binding protein; Gpp[NH]p, guanylyl-imidodiphosphate; IC_{50}, concentration of ligand causing 50% inhibition of specific receptor binding

2. MATERIALS AND METHODS

2.1. *Materials*

[^{3}H]yohimbine (79.5 Ci/mmol) was purchased from New England Nuclear/DuPont. Oxymetazoline, prazosin and noradrenaline were from Sigma. All reagents for tissue culture were purchased from Gibco (Paisley, Scotland).

2.2. *Cell culture*

Neuroblastoma × glioma hybrid, NG108-15, cells were grown in tissue culture as previously described [6]. In a number of cases the cells were treated with pertussis toxin (Porton Products, Porton Down, UK) (100 ng/ml, 16 h). Cells were harvested when some 80% confluent and membrane fractions prepared in 10 mM TrisHCl, 0.1 mM EDTA, pH 7.5 (buffer A) as in [6]. Membranes were stored at −70°C before use.

2.3. *Immunological studies*

The generation and specificities of the various antisera used in this

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

Table I

Generation and specificities of anti-G-protein antisera

Antiserum	Peptide employed	Corresponding G-protein sequence		Antiserum identifies
AS7	KENLKDCGLF	Transducin α	341–350	Transducin, G_i1, G_i2
OC1	ANNLRGCGLY	$G_o\alpha$	345–354	G_o
13B	KNNLKECGLY	$G_i3\alpha$	345–354	G_i3
CS1	RMHLRQYELL	$G_s\alpha$	385–394	G_s

study are defined in Table I. Each antiserum was produced in New Zealand White rabbit using a conjugate of a synthetic peptide and keyhole limpet haemocyanin (Calbiochem) as antigen. The details of this process have previously been recorded [7]. Details of the immunoblotting protocols and use of these antisera to study functional interactions of receptors and G-proteins have previously been recorded [4].

2.4. *Binding experiments*

Were performed at 25°C for 30 minutes in 10 mM Tris HCl, 50 mM sucrose, 20 mM $MgCl_2$, pH 7.5 (Buffer B). In saturation experiments using [^{3}H]yohimbine the concentration of ligand was varied between 0.5 and 20 nM. Non-specific binding was defined in all cases by parallel assays containing 100 μM noradrenaline. Non-specific binding increased with [^{3}H]ligand concentration in a linear manner. Binding experiments were terminated by rapid filtration through Whatman GF/C filters followed by three washes of the filter with ice cold buffer B (5 ml). In a number of experiments, the membranes were preincubated with IgG fractions derived from either normal rabbit serum of from a series of polyclonal anti-peptide antisera directed against the C-terminal decapeptides of the α subunit of the G-proteins expressed by NG108-15 cells (see below for production of the IgG fractions) for 60 min at 30°C before performing the binding assay. We have previously noted that such treatment will allow the uncoupling of receptors from G-proteins [4].

2.5. *Generation of IgG fractions from anti-G-protein antisera*

A column of protein A-sepharose 4B (Pharmacia) (0.5 ml) was equilibrated with 1.5 M glycine pH 8.9, 3 M NaCl (buffer C). 5 ml of antiserum in buffer C was added to the column and allowed to equilibrate with gentle mixing. The column was washed with buffer C until the eluate had $A_{280} \approx 0.0$ and then eluted with 100 mM citric acid, pH 4.0 into 2 M Tris/HCl, pH 7.5. The eluted IgG fractions were dialysed overnight against 1000 volumes of buffer A, then lyophilized and reconstituted with buffer A prior to use.

3. RESULTS

Saturation binding isotherms in which [^{3}H]yohimbine was used as a ligand for the α2 adrenergic receptor expressed by neuroblastoma × glioma hybrid, NG108-15, cells demonstrated an apparent single population of receptor sites for which the dissociation constant of [^{3}H]yohimbine was some 2.5 ± 0.6 nM (mean ± SD, $n = 3$) and maximal capacity some 80 ± 14 fmol/mg membrane protein (mean ± SD, $n = 3$) (results not shown). Displacement of specific [^{3}H]yohimbine binding from this site was achieved with high affinity by both prazosin (IC_{50} corrected for receptor occupancy = 33 ± 5 nM) and oxymetazoline (IC_{50} corrected for receptor occupancy = 24 ± 10 nM) (means ± S.D., $n = 3$) which suggests that the receptor is of the α2B subclass. Specific [^{3}H]yohimbine binding was displaced by noradrenaline (IC_{50} corrected for receptor occupancy = 0.5 μM) and the effectiveness of noradrenaline to inhibit specific [^{3}H]yohimbine binding was reduced some 5-fold (IC_{50} corrected for receptor occupancy = 2.6 μM) when the experiment was performed in the presence of Gpp[NH]p (100 μM). By immunoblotting membrane fractions of NG108-15 cells with a series of antipeptide antisera directed against the extreme C-terminal region of the various G-proteins we demonstrated the expression of the α subunits of each of G_i2,G_i3,G_o and G_s, as we have recorded previously [4]. However, $G_i1\alpha$ is either not expressed or its expression is so limited that it is below current levels of detection [4].

The apparent specific binding of a single concentration of [^{3}H]yohimbine (2 nM) in the presence of 1 μM noradrenaline was, in the experiment displayed in Fig. 1, 28% of that in the absence of noradrenaline. This was increased to 75% of the total specific binding when the experiment was performed in the presence of GPP[NH]p (100 μM) (Fig. 1). This difference in specific binding of [^{3}H]yohimbine when the experiment was performed in either the presence or absence of GPP[NH]p thus provides a measure of receptor-interaction with the G-protein population of these membranes.

When membranes from NG108-15 cells were preincubated with an IgG fraction isolated from antiserum AS7, which in these cells identifies only G_i2 [4], the level of specific binding of [^{3}H]yohimbine in the presence of 1 μM noradrenaline was similar to that when the binding assay was performed in the presence of Gpp[NH]p (70% of total specific binding) (Fig. 1). An IgG fraction from normal rabbit serum was unable to mimic this effect of antiserum AS7 (Fig. 1). By contrast, incubation of membranes with the IgG fraction from antiserum AS7 or in the presence of Gpp[NH]p had no effect on the total specific binding of [^{3}H]yohimbine (Fig. 2).

Following preincubation of cells with pertussis toxin (100 ng/ml, 16 h) and the preparation of membranes, specific [^{3}H]yohimbine binding in the presence of 1 μM noradrenaline was significantly greater (67 ± 3% of total specific binding, mean ± S.D. ($n = 4$)) than in membranes derived from untreated cells (40 ± 4%) (Table II). Neither Gpp[NH]p (100 μM] or preincubation with the IgG fraction from antiserum AS7 was able to modify the extent of displacement of specific [^{3}H]yohimbine binding by 1 μM noradrenaline from

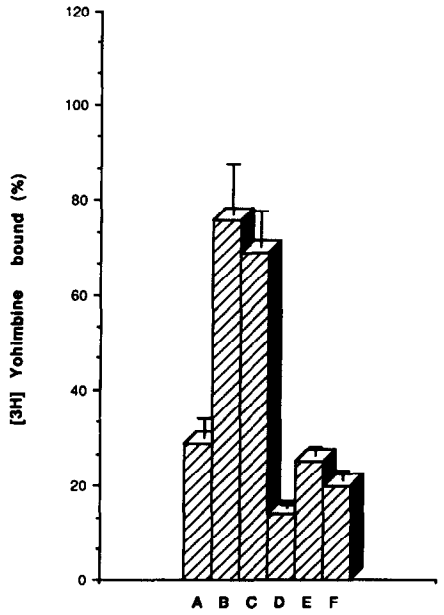

Fig. 1. Antibodies from antiserum AS7 but not from antisera directed against the C-terminal region of other G-proteins mimic Gpp[NH]p in causing an increase in the apparent specific binding of [^{3}H]yohimbine in the presence of 1 μM noradrenaline to membranes of NG108-15 cells. Membranes (100 μg) from undifferentiated NG108-15 cells were incubated with IgG fractions (amount equivalent to a 1:50 dilution of the crude antiserum) from either normal rabbit serum (A,B), antiserum AS7 (C), antiserum OC1 (D), antiserum CS1 (E) or antiserum 13B (F) for 60 min at 30°C. The specific binding of [^{3}H]yohimbine (2 nM) in the presence of 1 μM noradrenaline was then assessed as in section 2. During the binding assay Gpp[NH]p (100 μM) was also included in (B). In these experiments 100% [^{3}H]yohimbine specific binding was 24.0 fmol/mg membrane protein. Bars represent means ± SD (n = 4).

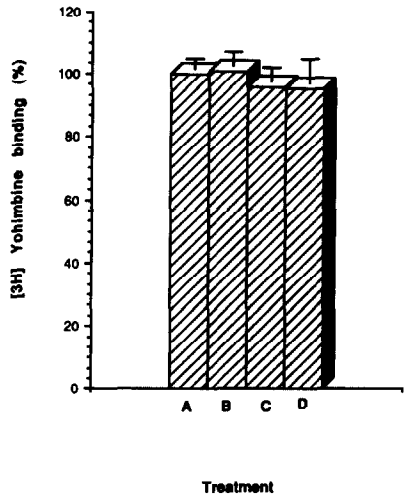

Fig. 2. Lack of effect of agents which uncouple α2 receptors from the G-protein signalling cascade on the total specific binding of [^{3}H]yohimbine. Membranes (100 μg) from undifferentiated NG108-15 cells were preincubated as in Fig. 3 with either no additions (A,D), an IgG fraction from antiserum AS7 (B) or an equivalent IgG fraction from normal rabbit serum (C). The specific binding of [^{3}H]yohimbine (2 nM) was then assessed as in section 2. During the binding assay Gpp[NH]p was also included in (D). In this experiment 100% specific binding was 23.9 fmol/mg protein. Bars represent means ± SD (n = 4).

membranes from pertussis toxin-pretreated cells in which the receptor is uncoupled from its G-protein (Table 2) in contrast to the effects of these agents in membranes from untreated cells (Fig. 1).

To assess the potential interactions of the α2 adrenergic receptor with other G-proteins, membranes of untreated NG108-15 cells were preincubated with IgG fractions isolated from antisera directed against the C-terminal decapeptides of each of the other G-proteins expressed by these cells ($G_o\alpha$ (OC1), $G_i3\alpha$ (13B) or $G_s\alpha$ (CS1)). None of these antibody preparations altered specific [^{3}H]yohimbine binding, whether measured in the presence or absence of 1 μM noradrenaline (Fig. 3 and results not shown) although all can immunoprecipitate the relevant G-proteins from membranes of these cells (results not shown).

4. DISCUSSION

In undifferentiated neuroblastoma x glioma hybrid,

Table II

Gpp[NH]p and antibodies from antiserum AS7 have no effect on the specific binding of [^{3}H]yohimbine in the presence of 1 μM noradrenaline in membranes from NG108-15 cells which have been treated with pertussin toxin

Treatment	[^{3}H]yohimbine binding in the presence of 1 μM noradrenaline (% total specific)
Untreated cells: preincubation with non-immune IgG	40 ± 4
Pertussis toxin pretreated cells: preincubation with non-immune IgG	67 ± 3
Pertussis toxin pretreated cells: preincubation with non-immune IgG Gpp[NH]p in binding assay	74 ± 12
Pertussis toxin pretreated cells: preincubation with IgG form antiserum AS7	67 ± 2

NG108-15 cells were treated with either pertussis toxin (100 ng/ml, 16 h) or with vehicle and membranes prepared. Binding assays were performed as in section 2 in either the presence or absence of Gpp[NH]p (100 μM) following preincubation with IgG fractions (amounts equivalent to a 1:50 dilution of the serum) isolated from either antiserum AS7 or from non-immune serum. Numbers represent means ± SD (n = 4).

NG108-15, cells an $\alpha 2$ adrenergic receptor is able to mediate inhibition of adenylate cyclase [8]. Pretreatment of the cells with pertussis toxin attenuates adrenergic inhibition of adenylate cyclase by preventing productive contact between receptor and its G-protein [5]. This indicates that receptor-mediated inhibition of adenylate cyclase is transduced by one or more members of the family of G-proteins which are substrates for mono-ADP-ribosylation catalysed by pertussis toxin. The primary sequence of each of these pertussis toxin sensitive G-proteins has a cysteine residue located 4 amino acids from the C-terminus and it is this amino acid which acts as the acceptor for pertussis toxin-catalysed ADP-ribosylation [9]. These observations provided the first evidence that the C-terminal region of G-proteins was likely to represent a key site for contact between receptors and G-proteins. We reasoned that antisera directed against the C-terminal region of the α subunit of various G-proteins might be able to uncouple receptors and G-proteins and would thus provide information of the specificity or otherwise of receptor-G-protein coupling in native membranes and in whole cells. We have generated a series of antipeptide antisera directed against the C-terminal decapeptides of various G-proteins and have previously characterized their specificity for the individual G-proteins in immunoblots and immunoprecipitation protocols [7,10,11–13]. We, and others, have been able to use such antisera to interfere with the interaction of receptors with each of G_s [14] and Nair, Parikh, Milligan and Patel (submitted) G_i2 [4,6,15], rod transducin [16] and G_o [17] by measuring the attenuation of either receptor-driven high affinity GTPase activity or of the activity of the effector system.

For technical reasons it is often difficult, however, to measure receptor-driven increases in high affinity GTPase activity [18]. Moreover, regulation of second messenger production is removed both spatially and temporally from the initial contact between receptor and G-protein and hence might be subject to extraneous influences. To combat such problems we have now developed an assay which is potentially amenable to all receptors which interact with G-proteins and which should allow assessment of the specificity (or otherwise) of receptor-G-protein interaction. This assay relies on the reduction in binding affinity for agonists but not antagonists, which has been well established to occur when receptor and G-protein become dissociated from one another [18].

In membranes of undifferentiated NG108-15 cells, in the absence of guanine nucleotides, 1 μM noradrenaline displaced some 70% of specifically bound antagonist ([^{3}H]yohimbine (2 nM). However, when the poorly hydrolysed GTP analogue, Gpp[NH]p (100 μM) was also present only 30% of the [^{3}H]yohimbine was displaced by 1 μM noradrenaline. We then assessed whether antipeptide anti-sera to the various G-proteins which are expressed by NG108-15 cells would be able to mimic the effect of Gpp[NH]p. Following preincubation of membranes of NG108-15 cells with antibodies derived from antiserum AS7, exactly the same increase of specific [^{3}H]yohimbine binding in the presence of noradrenaline (1 μM) was observed as with Gpp[NH]p (Fig. 1). Moreover, like Gpp[NH]p, the IgG fraction from antiserum AS7 had no effect on total specific [^{3}H]yohimbine binding (Fig. 2). By contrast, the IgG fractions derived from normal rabbit serum and from each of antisera 13B (anti-$G_i3\alpha$), OC1 (anti-$G_o\alpha$) and CS1 (anti-$G_s\alpha$) had no effect on either total specific binding of [^{3}H]yohimbine or specific binding of this ligand in the presence of 1 μM noradrenaline.

Antiserum AS7 was produced against the C-terminal decapeptide of rod transducin but identifies both $G_i1\alpha$ and $G_i2\alpha$ as well as transducin [7]. This reflects the fact that the C-terminal decapeptides of G_i1 and G_i2 are identical and differ in but a single amino acid from that of transducin. As such this antiserum cannot provide a selective probe for functional studies in cells or tissues in which more than one of these three polypeptides is expressed. The expression of transducin is limited to photoreceptor containing tissues and we have demonstrated previously that G_i1 is not expressed by NG108-15 cells [4]. This antiserum can therefore be used as a specific probe for G_i2 in these cells. Treatment of membranes of undifferentiated NG108-15 cells with antibodies from antiserum AS7, but not with equivalent antibodies which identify the other G-proteins present in the plasma membrane of these cells, converted the $\alpha 2$ adrenergic receptor to a form which displayed reduced affinity for agonist but not antagonist. Hence we are able to conclude that the $\alpha 2$ adrenergic receptor interacts directly with G_i2.

We have previously noted that $\alpha 2$ adrenergic inhibition of Ca^{2+} currents in NG108-15 cells is attenuated by intracellular injection of antibodies from antiserum OC1 (anti-$G_o\alpha$) and not by antibodies from either antiserum AS7 (anti-$G_i2\alpha$) or antiserum 13B (anti-$G_i3\alpha$) [17]. These data provided clear evidence that G_o functions to regulate receptor-control of voltage-sensitive Ca^{2+} channels in NG108-15 cells [17], a proposal which had originally been suggested by reconstitution experiments by Schultz and co-workers [19]. We must therefore address why it appears in the experiments reported herein, that $\alpha 2$ receptors interact exclusively with G_i2. The major difference in the experiments described here and those employing electrophysiological approaches is the state of the NG108-15 cells. For electrophysiological experimentation it is routine to chemically 'differentiate' the cells by treating them for a number of days with one of a range of agents which elevate intracellular cAMP levels [1]. During such 'differentiation' the cells withdraw from the cell cycle and become considerable more electrically excitable [1]. In parellel with the 'differentiation',

cellular levels of G_o increase markedly [11], although there is no reason to believe that these two phenomena are other than co-incidental. Dependent upon the agent employed to 'differentiate' the cells, levels of G_i2 may fall considerably or remain relatively unchanged [20, Mullaney, Frenz and Milligan, unpublished observations]. In the undifferentiated state, G_o appears to represent only some 5–10% of the total population of pertussis toxin sensitive G-proteins in the cells [17]. By contrast, in the 'differentiated' cells this G-protein represents between 25–50% of the total pertussis toxin-sensitive G-proteins. The two states are therefore not highly comparable. It is thus possible that the $\alpha 2$ adrenergic receptor is physically capable of interacting with two distinct G-proteins and that the relative proportions of these at the membrane will determine the integration of effector regulation following agonist activation of the receptor [17].

In previous studies we have demonstrated that the binding of a [^{3}H]agonist to a δ opioid receptor in NG108-15 cell membranes is reduced in affinity but not maximal capacity by pretreatment of membranes from the cells with antibodies from antiserum AS7 [4]. Here we observe an alteration in apparent specific receptor binding of a [^{3}H]antagonist produced by this same antibody. This effect is based upon competition for the receptor between the [^{3}H]antagonist and a fixed concentration of a receptor agonist. We propose that such approaches should be useful to study the specificity of interactions of any receptor with a G-protein.

Acknowledgements: These studies were supported by project grants from the Medical Research Council (UK) to G.M.

REFERENCES

[1] Hamprecht, B., Glaser, T., Reiser, G., Bayer, G. and Probst, F. (1985) Meth. Enzymol. 109, 316–341.
[2] Milligan, G., McKenzie, F.R., McClue, S.J., Mitchell, F.M. and Mullaney, I. (1990) Int. J. Biochem. (in press).
[3] Katada, T. and Ui, M. (1982) J. Biol. Chem. 257, 7210–7216.
[4] McKenzie, F.R. and Milligan, G. (1990) Biochem. J. 267, 371–378.
[5] Kurose, H., Katada, T., Amano, T. and Ui, M. (1983) J. Biol. Chem. 258, 4870–4875.
[6] McKenzie, F.R., Kelly, E.C.H., Unson, C.G., Spiegel, A.M. and Milligan, G. (1988) Biochem. J. 249, 653–659.
[7] Goldsmith, P., Gierschik, P., Milligan, G., Unson, C.G., Vinitsky, R., Malech, H. and Spiegel, A.M. (1987) J. Biol. Chem. 262, 14683–14688.
[8] Sabol, S.L. and Nirenberg, M. (1979) J. Biol. Chem. 254, 1913–1920.
[9] Lochrie, M.A. and Simon, M.I. (1988) Biochemistry 27, 4957–4965.
[10] Mitchell, F.M., Griffiths, S.L., Saggerson, E.D., Houslay, M.D., Knowler, J.T. and Milligan, G. (1989) Biochem. J. 262, 403–408.
[11] Mullaney, I. and Milligan, G. (1989) FEBS Lett. 244, 113–118.
[12] Milligan, G. and Unson, C.G. (1989) Biochem. J. 260, 837–841.
[13] Bushfield, M., Murphy, G.J., Lavan, B.E., Parker, P.J., Hruby, V.J., Milligan, G. and Houslay, M.D. (1990) Biochem. J. 268, 449–457.
[14] Simonds, W.F., Goldsmith, P.K., Woodward, C.J., Unson, C.G. and Spiegel, A.M. (1989) FEBS Lett. 249, 189–194.
[15] Simonds, W.F., Goldsmith, P.K., Codina, J., Unson, C.G. and Spiegel, A.M. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 7809–7813.
[16] Cerione, R.A., Kroll, S., Rajaram, R., Unson, C., Goldsmith, P. and Spiegel, A.M. (1988) J. Biol. Chem. 263, 9345–9352.
[17] McFadzean, I., Mullaney, I., Brown, D.A. and Milligan, G. (1989) Neuron 3, 177–182.
[18] Milligan, G. (1988) Biochem. J. 255, 1–13.
[19] Hescheler, J., Rosenthal, W., Trautwein, W. and Schultz, G. (1987) Nature 325, 445–447.
[20] Mullaney, I., Magee, A.I., Unson, C.G. and Milligan, G. (1988) Biochem. J. 256, 649–656.