Volume 229, number 1, 131–134 FEB 05621 February 1988

Block of light responses of salamander rods by pertussis toxin and reversal by nicotinamide

G. Falk and R.A. Shiells

Biophysics Unit, Department of Physiology, University College London, Gower Street, London WC1E 6BT, England

Received 23 December 1987

Transducin is the substrate for a pertussis toxin-catalyzed ADP-ribosylation in isolated retinal rod disk membranes [(1984) J. Biol. Chem. 259, 23–26]. The effects of the toxin on the light responses of intact dark-adapted rods were studied. Applied close to a rod outer segment in a retinal slice, pertussis toxin depolarized the rod by a few millivolts and produced a long-lasting depression of light responses, effects which depended on penetration of toxin into rods. Nicotinamide, an inhibitor of ADP-ribosylation, not only blocked the action of the toxin, but also reversed the effects once established. The action of nicotinamide itself on rods indicates the presence of endogenous ADP-ribosyltransferases which may constitute a control system modulating phototransduction. Inhibition of phospholipase C by neomycin had only transient effects indicating that the cGMP, rather than a phosphoinositide, pathway is primary in vertebrate phototransduction. Rapid reversal of pertussis toxin action suggests possible clinical applications of nicotinamide or congeners to the treatment of disease caused by ADP-ribosylating bacterial toxins.

Phototransduction; Pertussis toxin; Transducin; ADP-ribosylation; Nicotinamide; Phospholipase C

1. INTRODUCTION

GTP-binding proteins play a central role in signal transduction, involving control of second messengers in hormonal regulation, neurotransmission and vision [2,3]. Photoactivation of rhodopsin in retinal rods initiates an amplifying enzymic cascade involving the G-protein, transducin, leading to activation of a cGMP phosphodiesterase [3]. cGMP has the requisite properties of the internal transmitter which opens cation channels [4,5]. Hence, light results in a closure of channels and a consequent hyperpolarization (an increase in internal negativity) of the rod. The light-activated cascade can be inhibited in isolated rod disk membrane by pertussis toxin, one of a group of ADP-ribosylating bacterial toxins with an A5B structure, where A is a protomer containing the catalytic fragment and B is a pentameric peptide [6]. The toxin is thought to enter cells after the B-subunit binds to a receptor on the cell surface and allows the entry of the A-subunit [7]. Inside the cell the catalytic fragment of the A-subunit is activated by the disulphide bond reduction [8].

Light also induces hydrolysis of phosphatidylinositol bisphosphate [9]. It was therefore important to compare the effects on rods of inhibition of the cGMP cascade with inhibition of phospholipase C.

Correspondence address: G. Falk, Biophysics Unit, Department of Physiology, University College London, Gower Street, London WC1E 6BT, England

2. MATERIALS AND METHODS

Intracellular voltage recordings were made from the outer segments of dark-adapted rods in 150–200 μm thick radial slices prepared from axolotl (*Ambystoma mexicanum*) retinae. The slices and electrodes were viewed under infrared light by means of a microscope and TV camera. The bathing solution was a modified Leibovitz L-15 culture medium [10] with major constituents (mM): Na^+, 107; Cl^-, 114; K^+, 2.4; Ca^{2+}, 3.6; Mg^{2+}, 0.7; glucose, 5; Hepes, 5; pH 7.6. After a rod was penetrated, dim light flashes, about 35 ms in duration, were applied every 10 s.

Pertussis toxin was applied by a brief (200 ms) pressure (100 mmHg) pulse to a pipette positioned close to the recording

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

site. The pipette contained 88 μg/ml of *Bordetella pertussis* toxin (Porton Products) in 280 mM NaCl plus 0.8-times the concentration of the other constituents of a modified synthetic culture medium and 10 mM Tris, pH 8. A test flash was omitted at the time of ejection. In some experiments the toxin was pretreated for 2 h with 1 mM dithiothreitol at about 20°C. Toxin heated at 100°C for 10 min was without any effect and this test also served as a control for the inorganic constituents (such as the elevated NaCl concentration) in the pressure pipette.

Nicotinamide was applied to a rod outer segment either by a brief pressure pulse (0.2 M nicotinamide in culture medium in the pipette) or by replacement of the bathing solution by one with nicotinamide added. Neomycin sulphate (0.4 M in the pipette) was injected ionophoretically into a rod outer segment through the same electrode used for voltage recording.

3. RESULTS

Typical effects of the holotoxin, applied close to the external surface of a rod outer segment in a retinal slice, are shown in fig.1A. After a lag of 30–60 s, there was a slow and prolonged depolarization of about 5 mV and a gradual decline in sensitivity to dim and to bright flashes, as well as to steps of light (not shown). Responses to bright flashes were prolonged, although the time course of the initial part of the light-induced hyperpolarization was unaffected (fig.1A, lower set of records).

The decline in light responses cannot be ascribed to the depolarization, which in any case was fairly modest and represented only a small fraction of the driving force for the response, assuming a reversal potential of −5 mV. Furthermore, the decline in light responses was somewhat slower than the time course of depolarization and the light responses continued to decline when there was no further change in the dark potential. In a few cells it was possible to record stably over a long period of time and to observe a slow repolarization which began some 15–20 min after application of toxin. However, light responses failed to recover.

That the action of pertussis toxin depended on penetration into rods was shown by the lack of effect of externally applied toxin which had been dissociated by dithiothreitol (fig.1B). This treatment releases the catalytic fragment of the toxin but interferes with entry into intact cells [8]. The relatively rapid action of the toxin may reflect a high density of surface receptors for entry and the condensed lipid structure of internal disk membranes favourable for the activity of toxin with its substrate.

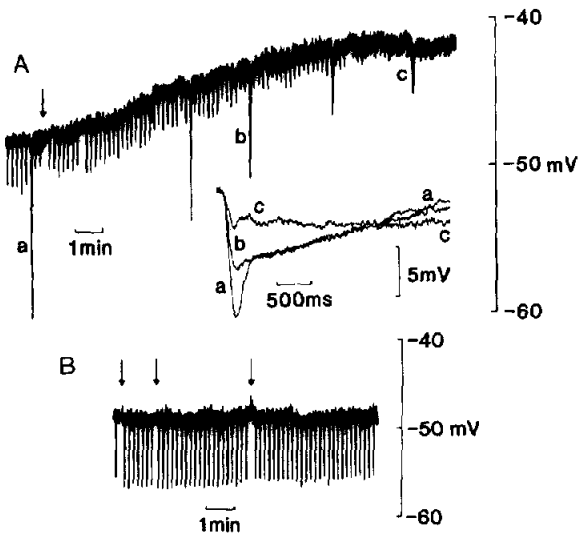

Fig.1. Effect of pertussis holotoxin (A) applied outside a rod outer segment in a retinal slice and the ineffectiveness of toxin dissociated by dithiothreitol (B). (A) Intracellular recording from a rod outer segment. Dim light flashes were applied every 10 s. At intervals, the light intensity was increased to give a near maximal response (as at a,b,c, also shown on an expanded sweep in the lower part of A). Pertussis toxin was applied as a brief pulse (arrow) to a pipette positioned close to the recording site. (B) Another experiment with the same protocol as A, but the toxin (arrows) was pretreated with dithiothreitol.

It was important to determine whether the action of pertussis toxin was due to ADP-ribosylation of transducin, the major G-protein of rod outer segments. Two sets of experiments were performed. Nicotinamide, an inhibitor of ADP-ribosylation [11,12], was ejected as a pulse from a pipette close to a dark-adapted rod outer segment. Nicotinamide, which is uncharged, readily penetrates cells. Nicotinamide hyperpolarized the cell by about 4 mV and interestingly, increased flash sensitivity (fig.2A). The rod no longer responded to test pulses of pertussis toxin for about 5–10 min, but then gradually lost light responses and slowly depolarized by a few millivolts, presumably as nicotinamide diffused out of the rod. In another set of experiments, rods were treated with pertussis toxin and the effects of the toxin on light responses and dark potential allowed to develop (fig.2B). The solution bathing the retinal slice was then exchanged for one containing nicotinamide. This resulted in restoration of light responses and hyperpolarization of the cell, to a

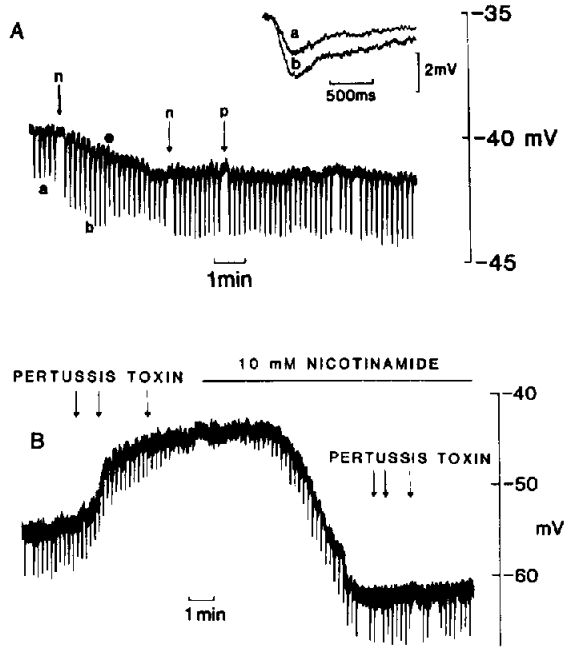

Fig.2. Block of pertussis toxin action (A) and reversal of the effects of the toxin by nicotinamide (B). (A) Nicotinamide was applied to a rod outer segment by a brief pressure pulse at n, pulses of pertussis toxin given at p. The flash responses marked a,b are shown superimposed on an expanded time scale in the inset. Because of the increase in light responses with nicotinamide, test flash intensity was decreased from the point indicated by ● onwards. About 5 min after the last test pulse of pertussis toxin, the cell gradually depolarized and light responses diminished (not shown). (B) Pulses of pertussis toxin applied at the arrows followed later by replacement of the bathing solution by one to which 10 mM nicotinamide was added.

potential more negative than the normal dark potential, this level being dependent on the nicotinamide concentration. Further applications of pertussis toxin were ineffective. The conclusion from these experiments is that the effects of pertussis toxin are a result of ADP-ribosylation, most likely of a G-protein.

In order to determine whether the inhibition of light responses by toxin was due to inactivation of the cGMP cascade or to ADP-ribosylation of a G-protein linked to a light-induced activation of phospholipase C, yielding inositol trisphosphate and diacylglycerol from polyphosphoinositide [9,13] neomycin, an inhibitor of phospholipase C, was injected ionophoretically into rod outer segments. Neomycin is a polyvalent cation which does not cross cell membranes. Neomycin caused only a transient depolarization and a very transient decrease in flash response, quite different from the effects of pertussis toxin. We conclude that the effects of the toxin follow from ADP-ribosylation of transducin and that the cGMP enzymic cascade is the major pathway in phototransduction, although these experiments do not exclude some regulatory role for a phosphoinositide pathway.

5. DISCUSSION

In terms of the transducin cGMP cascade, the inhibition of light responses by pertussis toxin would be anticipated. The depolarization of the cell by pertussis toxin, which probably results from an increase in the steady-state concentration of cGMP, remains to be explained and raises the important question of the control of cGMP concentration in the dark. It has been reported from studies with ^{18}O in the rabbit retina [14] that there is a rapid hydrolytic flux of cGMP in the dark reflecting phosphodiesterase and guanylate cyclase activity with a half-turnover time of only 1.5 s. The exchange of GTP for GDP is extremely slow in the dark but not negligible [15]. The α-subunit of transducin bound to GTP activates phosphodiesterase and the presence of photoexcited rhodopsin is not required for this step in the cascade [3]. The observation that ADP-ribosylation of transducin by pertussis toxin decreases the binding of a stable analogue of GTP [16] may provide an explanation for the pertussis toxin-induced depolarization in the dark. A decrease in the relative affinities of the guanyl nucleotides or further occlusion of the binding site [16] would decrease T_α-GTP and phosphodiesterase activity in the dark. A 10–15% decrease in the GTPase activity of transducin treated with pertussis toxin, evident in fig.2 of [1], would be consistent with the above explanation. This decrease may be of the correct magnitude to explain the fairly modest depolarization observed taking into account that the effect on the fraction of channels open in the dark could be amplified if the free cGMP concentration were in the range where a power-law applied [4,17].

The hyperpolarization of dark-adapted rods by nicotinamide may indicate the presence of an endogenous ADP-ribosyltransferase involved in the

control of the concentration of cGMP in the dark. An ADP-ribosyltransferase has been isolated from rod outer segments [18]. We have recently observed that the effect of nicotinamide on membrane potential of rods depends on their light adaptational state [19] which raises the intriguing possibility of endogenous transferases with the substrate specificities of pertussis and cholera toxins, corresponding to the dark and light states of transducin, respectively [1]. The reversal of ADP-ribosylation by nicotinamide suggests a possible clinical application to diseases caused by bacterial ADP-ribosylating toxins.

Acknowledgements: We thank the Medical Research Council for a project grant and Professor B. Gomperts and Dr J. Mowbray for valuable suggestions.

REFERENCES

[1] Van Dop, C., Yamanaka, G., Steinberg, F., Sekura, R.D., Manclark, C.R., Stryer, L. and Bourne, H.R. (1984) J. Biol. Chem. 259, 23–26.
[2] Cohen, P. and Housley, M.D. (1985) Molecular Mechanisms of Transmembrane Signalling, Elsevier, Amsterdam, New York.
[3] Stryer, L. (1986) Annu. Rev. Neurosci. 9, 87–119.
[4] Fesenko, E.E., Kolesnikov, S.S. and Lyubarsky, A.L. (1985) Nature 313, 310–313.
[5] Matthews, G. and Watanabe, S.-I. (1987) J. Physiol. 389, 691–715.
[6] Tamura, M., Nogimori, K., Yajima, M., Ase, K. and Ui, M. (1983) J. Biol. Chem. 258, 6756–6761.
[7] Tamura, M., Nogimori, K., Murai, S., Yajima, M., Ito, K., Katada, T., Ui, M. and Ishii, S. (1982) Biochemistry 21, 5516–5522.
[8] Katada, T., Tamura, M. and Ui, M. (1983) Arch. Biochem. Biophys. 224, 290–298.
[9] Ghalayini, A. and Anderson, R.E. (1984) Biochem. Biophys. Res. Commun. 124, 503–506.
[10] Attwell, D. and Wilson, M. (1980) J. Physiol. 309, 287–315.
[11] Chung, D.W. and Collier, R.J. (1977) Biochim. Biophys. Acta 483, 248–257.
[12] Katada, T. and Ui, M. (1982) J. Biol. Chem. 257, 7210–7216.
[13] Berridge, M. and Irvine, R.F. (1984) Nature 312, 315–321.
[14] Goldberg, N.D., Ames, A. iii, Gander, J.E. and Walseth, T.F. (1983) J. Biol. Chem. 258, 9213–9219.
[15] Bennett, N. and Dupont, Y. (1985) J. Biol. Chem. 260, 4156–4168.
[16] Bruni, P., Hewlett, E.L. and Moss, J. (1985) Biochem. Biophys. Res. Commun. 127, 999–1006.
[17] Yau, K.-W. and Nakatani, K. (1985) Nature 317, 252–255.
[18] Schmidt, G.J., Wallis, C.J., Huber, L.J. and Weiter, J.J. (1987) Invest. Ophth. vis. Sci. 28, 94.
[19] Falk, G. and Shiells, R.A. (1988) J. Physiol. 396, 128P.