Pertussis toxin prevents the inhibitory effect of adenosine and unmasks adenosine-induced excitation of mammalian motor nerve endings

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Pertussis toxin (PTX), which blocks certain classes of guanine nucleotide binding proteins (G proteins), consistently blocked the inhibitory effects of adenosine (100 μ M–250 μ M) on quantal acetylcholine (ACh) secretion in rat phrenic nerve hemidiaphragm preparations. PTX pretreatment also highlighted long-lasting increases in evoked ACh release elicited by adenosine. The results suggest that specific G proteins are involved in mediating the inhibitory effects of adenosine at motor nerve endings.

Introduction Studies on the mechanism by which adenosine receptor binding is transduced into the inhibition of neurotransmitter secretion are replete with controversy at present (see e.g. Ribeiro & Sebastiao, 1986; Silinsky, 1986; Fredholm & Dunwiddie, 1988). At motor nerve endings, it has been suggested that a guanine nucleotide binding protein (G-protein, see Graziano & Gilman, 1987) is an important transducer of inhibitory effects of adenosine (Silinsky, 1985, Figure 4). As a means of testing this hypothesis, it should be possible to inactivate several types of G proteins at motor nerve endings by ADP-ribosylating their alpha subunits with pertussis toxin (PTX; Graziano & Gilman, 1987). If PTX were observed to occlude the inhibitory action of adenosine, then such a result would be consistent with the suggestion that certain classes of G proteins are involved in coupling adenosine receptor binding to inhibition of ACh secretion.

Methods Conventional methods for intracellular recording and stimulation were employed in rat phrenic nerve hemi-diaphragm preparations (see Gage & Hubbard, 1966). For further details of the

methods for nerve stimulation, electrophysiological recording and data analysis, see Silinsky (1987).

In these studies, Male-Sprague Dawley rats of 128–200 g were injected with 10 μ g of Islet Activating Protein (pertussis toxin, List Biological Laboratories) in 500 μ l of physiological saline + bovine serum albumin (1 mg ml⁻¹). Four–nine days after injection, the diaphragms were excised and studied *in vitro*.

Results Figure 1a illustrates the typical effect of 250 μ M adenosine on preparations from control animals, namely a maximal inhibition of evoked ACh secretion (reflected as endplate potentials; e.p.ps) to approximately 50% of the control level. In contrast, when this concentration of adenosine was applied to preparations from rats injected with PTX, no inhibitory effect was observed during adenosine application (Figure 1b). The inability of adenosine (100–250 μ M) to inhibit release after PTX-treatment was reproduced in all 8 experiments using the protocol described in the methods. In contrast to adenosine, the inhibitory effect of Mg, which exerts a direct blocking effect on the Ca channel, was unaffected by PTX treatment.

Whilst no inhibitory effect of adenosine was observed in PTX-treated preparations, rebound increases in evoked ACh release occurred after the cessation of superfusion with adenosine in such preparations (e.g. Figure 1b). Furthermore, in the experiment illustrated, subsequent addition of 250 μ M adenosine failed to alter ACh release from its increased level in the PTX-treated preparation. The rebound increases in ACh release ranged from 1.4 to 2.7 times the control level ($n = 8$) with a mean increase of 1.9 fold over control ($n = 8$).

In two experiments in which accurate measurements of miniature endplate potentials (m.e.p.ps) were possible, the effects of adenosine on spontaneous ACh release, i.e., m.e.p.p. frequency, were also studied. Normally adenosine produces a parallel fall

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Figure 1 Comparison of the effects of adenosine ($250 \mu\text{M}$) in control (a) and in diaphragms from rats treated with pertussis toxin (PTX) (b). Each symbol represents the average of 30–32 e.p.ps (for statistical methods see Silinsky, 1987). As adenosine does not alter the post-junctional acetylcholine (ACh) sensitivity, changes in e.p.p. amplitudes accurately reflect changes in ACh release. (□) Control; (▲) $250 \mu\text{M}$ adenosine. The physiological saline solution consisted of (mM): NaCl 137, KCl 5, CaCl_2 1, MgCl_2 20–25, NaH_2PO_4 1, NaHCO_3 24, glucose 11, bubbled with 95% O_2 : 5% CO_2 to pH 7.2 (see Gage & Hubbard, 1966). The Mg concentration was 25 mM in (a) and 20 mM in (b). The phrenic nerve was stimulated at a frequency of 0.1 Hz. Corrections for non-linear summation of e.p.ps were not required (see Silinsky, 1987). All experiments were done at room temperature. A large number of experiments were also done on frog cutaneous pectoris preparations using various protocols of injection and direct superfusion of isolated tissues with PTX. However, consistent results such as those found in the rat were not observed with frog preparations.

in evoked and spontaneous release (see references in Silinsky, 1986). After PTX treatment, however, no significant inhibitory effect of $250 \mu\text{M}$ adenosine on m.e.p.p. frequency was observed in these two experiments.

In the present studies, the level of Mg or curare needed for neuromuscular blockade was of a similar magnitude to that required by the untreated control

preparations. Unfortunately, variability in the Mg composition of solutions and in the degree of curarization required to reduce the e.p.p. below threshold in control and in PTX-treated preparations prevented a detailed comparison of ACh release in control animals and in pertussis-treated rats.

Discussion The results demonstrate two features of note after PTX-treatment of rat motor nerve endings. The first is the elimination of the inhibitory effect of adenosine by PTX and the second is the unmasking of increases in ACh release after adenosine application.

With respect to the inhibition of the presynaptic effects of adenosine by PTX, the results are in accordance with those of Dolphin & Prestwich (1985) who studied glutamate overflow from cultured cerebellar neurones and of O'Regan & Phillis (1987) who studied the firing of cortical neurones (but see Fredholm & Dunwiddie, 1988). At motor nerve endings, these present studies of quantal ACh release argue in favour of coupling of adenosine receptors to GTP binding proteins in mediating the inhibitory effects of adenosine. Specifically, the results suggest that adenosine receptors are linked to either G_i (the inhibitory G protein negatively coupled to adenylate cyclase), G_o (a G protein that may be linked to Ca-dependent events), or an as yet unidentified G protein.

Since it has been proposed that adenosine reduces the intracellular apparent affinity for Ca of a strategic component of the secretory apparatus (Silinsky, 1986), it is of interest that a recent report has shown a direct coupling between G_i or G_o and the active site of calcium binding proteins (Katada *et al.*, 1987). Specifically, Katada *et al.* (1987) showed Calmodulin binding to the beta/gamma subunits of PTX-sensitive G proteins. Adenosine, by increasing the free concentration of the beta/gamma subunits, might be expected to remove Ca binding proteins from participation in the secretory process.

The results also reduce the likelihood that G_s (and thus cyclic AMP elevation) is an important mediator of the inhibitory effects of adenosine in the rat. While some evidence is in support of the involvement of G_s in the action of adenosine has been provided in frog (Silinsky, 1986), the antagonism by PTX of the effect of adenosine would argue against an obligatory involvement of G_s , unless, as suggested by Cerione *et al.* (1985), active G_i is necessary for optimal activation of the G_s /adenylate cyclase system.

In summary the results implicate a PTX-sensitive G-protein in mediating the inhibitory effect of adenosine and a second, PTX-insensitive process for mediating the long-lasting increases in secretion provoked by adenosine. These effects occur without

occlusion by PTX of Ca-dependent depolarization-secretion coupling.

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