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Short Communications

Intrahippocampal injection of pertussis toxin blocks adenosine suppression of synaptic responses

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Adenosine exerts prominent inhibitory effects on synaptic transmission via a presynaptic action. Using the hippocampal slice preparation, we have found in electrophysiological experiments that this action of adenosine is blocked by intrahippocampal injections of pertussis toxin. In biochemical studies, we have confirmed that this treatment affects the GTP-binding proteins, G_i and G_o, in this preparation. These results indicate that both pre- and postsynaptic actions of adenosine involve pertussis toxin-sensitive GTP-binding proteins.

Adenosine is a potent modulator of neuronal activity in many brain areas[6,26]. Its inhibitory effects have been attributed both to inhibition of calcium conductances[4,17,18,20] and activation of potassium channels[15,28]. Many of adenosine's actions are sensitive to pertussis toxin treatment[3,5,19,28] indicating that G-proteins inactivated by this toxin are involved in coupling adenosine receptors to these ion channels.

Adenosine's actions have been particularly well characterized in the hippocampal slice preparation where it acts both pre- and postsynaptically[7,11,21–25]. Adenosine hyperpolarizes CA1 pyramidal neurons by activation of potassium channels. This action is thought to be mediated by G-proteins as adenosine's activation of potassium channels in neurons[6,28] and atrial cells[15] is blocked by pertussis toxin as is activation of similar potassium channels by serotonin and GABA in CA1 pyramidal neurons[2].

Adenosine also suppresses synaptic activation of CA1 pyramidal neurons and dentate granule cells. This action appears to stem primarily from a poorly understood presynaptic effect of adenosine leading to a reduction in transmitter release[10,21]. To ascertain whether a pertussis toxin-sensitive G-protein also mediates this action of adenosine, we examined adenosine suppression of synaptic responses in the hippocampal slice preparation after pertussis toxin treatment in vivo.

Adult male albino rats (150–250 g, male, Sprague–Dawley) were anesthetized with chloral hydrate (400 mg/kg, i.p.) and the head positioned in a stereotaxic apparatus. A burr hole was drilled over the dorsal hippocampus unilaterally and pertussis toxin (0.1–2.0 μg in 1–2 μl of 0.9% NaCl) was injected through a Hamilton syringe into the dorsal hippocampus using the following coordinates (2.0 L, 3.0 P, 3.0 V, incisor bar at –3).

For ADP-ribosylation assays, hippocampal slices were prepared using standard techniques[29]. Slices of 400 μm were obtained from adult male rats and maintained prior to recording in an interface chamber at room temperature. In animals treated with pertussis toxin, slices were taken from the dorsal half

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of the hippocampus as that contained the injection site. Electrophysiological recordings were made from submerged slices at 30 °C. The standard physiological saline was saturated with 95% O_2/5% CO_2 and consisted of (in mM): NaCl 130, KCl 5.0, $CaCl_2$ 2.5, $MgSO_4$ 1.5, NaH_2PO_4 1.2, $NaHCO_3$ 24, and glucose 10. The recording chamber provides constant perfusion and allows switching between salines with a valve. Field potential recordings were made from the CA1 pyramidal cell layer or the granule cell layer of the dentate gyrus with fiber-filled glass microelectrodes filled with 2 M NaCl having impedances of 3–10 MΩ at 135 Hz. For recordings in CA1, orthodromic field potentials were elicited every 10 s with 50 μs pulses from a bipolar stimulating electrode placed in the vicinity of the stratum radiatum. For studies of the dentate, orthodromic responses were elicited every 15 s with an electrode positioned in the molecular layer. Stimulation voltage was adjusted to a level just below that which produced a maximum population spike (PS) amplitude. Traces were stored on a Nicolet 2090 digital oscilloscope and recorded on a Gould 60000 X-Y plotter. Intracellular recordings from CA1 pyramidal neurons were made with glass fiber-filled microelectrodes filled with 2 M $KMeSO_4$, 10 mM HEPES having impedances of 40–80 MΩ at 135 Hz. Synaptically induced excitatory postsynaptic potentials (EPSPs) were stored on a Nicolet 4094 digital oscilloscope and plotted on a Hewlett-Packard XY plotter. Resting membrane potential was continuously recorded on a Gould 220 chart pen recorder. Hyperpolarizing current pulses of 0.1 nA were injected through the recording electrode to monitor membrane resistance.

For ADP-ribosylation assays hippocampal slices were stored at –20 °C in 50 mM Tris (pH 8.0), 1 mM EDTA, and 1 μg/ml soybean trypsin inhibitor. Upon thawing, each slice was sonicated in 500 μl of the same buffer, and the membranes separated by centrifugation (12,000 rpm for 20 min in an Eppendorf centrifuge 5414). Membrane pellets were resuspended by sonication in Buffer A, containing 100 mM Tris (pH 8.0), 5 mM EDTA, 2.5 mM isoniazid.

Pertussis toxin was activated by combining equal volumes of 10% w/v pertussis toxin (List Biochemicals, Campbell, CA) in Buffer A with 50 mM DTT, followed by incubation at 25 °C for 30 min. The ADP-ribosylation mixture, containing 30 μl of membranes in Buffer A, 30 μl of activated pertussis toxin (1 μg toxin per sample), and 15 μl of 6.8 μM [^{32}P]NAD (spec. act. 29.3 Ci/mmol; 1 mCi = 37.0 mBq) was incubated at 25 °C for 90 min. Membrane proteins were then separated on a 10% polyacrylamide/sodium dodecyl sulphate gel[16], and the dried gel was exposed to Kodak XAR-5 film.

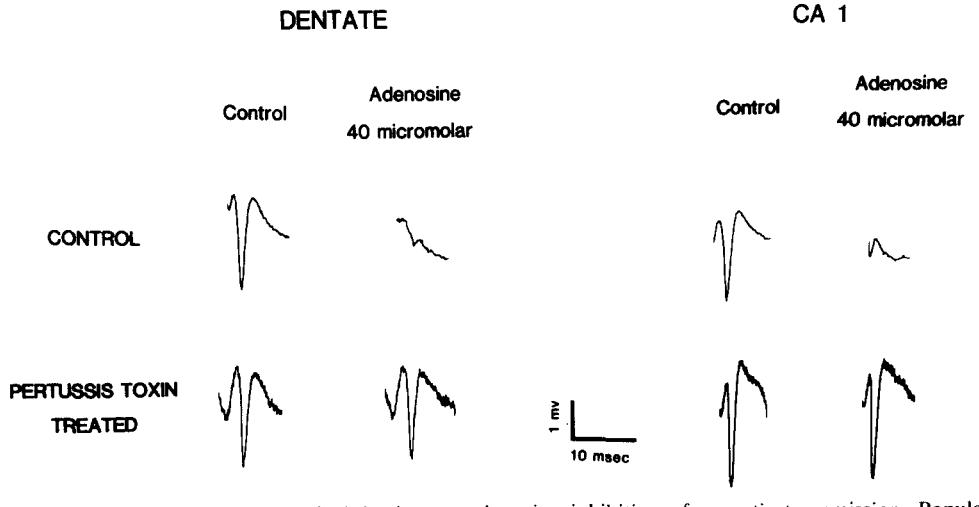

Fig. 1. Effect of intrahippocampal pertussis toxin injection on adenosine inhibition of synaptic transmission. Population spikes recorded from cell body layers of both CA1 and the dentate from control rats are abolished by 40 μM adenosine. After intrahippocampal injection with 1 μg pertussis toxin population spikes in both CA1 and the dentate are resistant to 40 μM adenosine. Each pair of traces is from a different slice.

EEG recordings were obtained using chronically implanted epidural stainless-steel screw electrodes. Tracings of bipolar derivation between frontal and parietal electrodes over each hemisphere were collected. Toxin injections were made at the same time as electrode implantation.

Hippocampal slices were prepared from the dorsal half of the hippocampus 3–8 days following intrahippocampal injection of pertussis toxin. In preliminary experiments, we found that injections of 0.1–0.5 μg of pertussis toxin did not reliably block adenosine's suppression of the orthodromic population spike. However, following treatment with 1–1.5 μg of pertussis toxin, adenosine reduced the population spike amplitude by only 7.7 ± 5% (mean ± S.E.M., 15 slices tested from 10 animals) at a concentration of 80 μM (Fig. 1) that completely suppresses this response in slices from control animals. Doubling the concentration of adenosine had no further effect in slices from pertussis toxin treated animals. Slices from animals treated with 1 μg pertussis toxin for only 24 or 48 h were not clearly affected. This delayed response to pertussis toxin is similar to that observed by other investigators and presumably reflects the time required for internalization of pertussis toxin and covalent modification of G-proteins in vivo[1,2,13].

Caffeine's ability to potentiate synaptic responses is thought to reflect blockade of adenosine receptors[12]. In some slices where the CA1 population spike was not reduced at all by 80 μM adenosine, the effect of caffeine was also examined. In these slices, caffeine (50–300 μM, n = 3) did not increase a submaximal population spike response as is typically observed in untreated slices (Fig. 2). These observations provide further evidence that caffeine's enhancement of synaptic responses reflects its blockade of the inhibitory action of endogenous adenosine.

Although adenosine's block of synaptic transmission is thought to reflect primarily a presynaptic decrease in transmitter release, at the concentrations used in this study, adenosine has significant hyperpolarizing effects postsynaptically. In order to con-

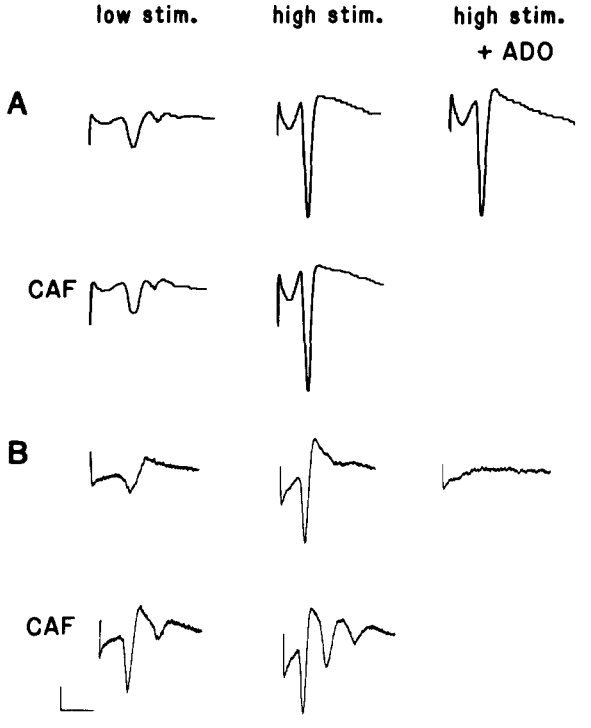

Fig. 2. Effect of pertussis toxin on caffeine responses in CA1. A: in a hippocampal slice from a rat injected with pertussis toxin, 40 μM adenosine (ADO) has no effect on a population spike recorded in CA1. Caffeine (CAF, 100 μM) also is without effect on population spikes elicited by either low or high stimulation strengths. B: in a hippocampal slice from a control rat, ADO (40 μM) completely suppresses synaptic transmission in CA1, and CAF (100 μM) increases the population spike amplitude at low stimulation strengths and causes multiple population spikes at high stimulation strengths. Calibration bars are 1 mV vertical, 5 ms horizontal.

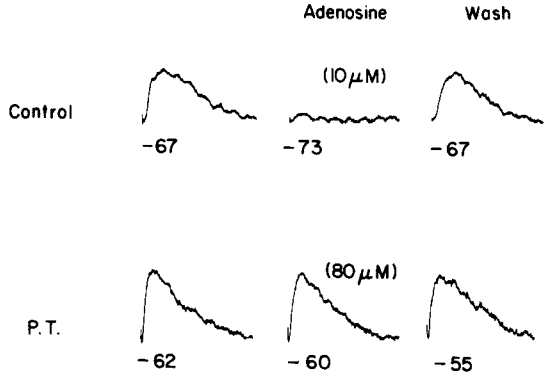

Fig. 3. Effect of pertussis toxin on pre- and postsynaptic actions of adenosine. EPSPs recorded from CA1 pyramidal cells induced by stimulation of Schaffer collaterals are blocked by low concentrations of adenosine in a control cell. The block of the EPSP is accompanied by hyperpolarization and decreased membrane resistance. Injection of 1.5 μg pertussis toxin 4 days previously blocks the effects of adenosine on both the EPSP and resting membrane potential and resistance. Resting membrane potential is listed under each tracing. Calibration is 20 ms horizontal, 2 mV vertical.

firm that adenosine's presynaptic actions are blocked by pertussis toxin treatment, intracellular recordings of synaptically induced EPSPs were recorded. In two CA1 neurons from 2 rats treated with 1.5 μg pertussis toxin 3 or 4 days previously, even 80 μM adenosine did not affect the resting membrane potential membrane input resistance, or synaptically induced EPSPs, whereas in untreated animals, 5–10 μM adenosine is sufficient to cause hyperpolarization, decreased membrane resistance and total suppression of EPSPs (Fig. 3).

In a few experiments, we also examined adenosine responses in the dentate granule cells. The effects of pertussis toxin were more variable with blockade of adenosine apparent in slices from half the animals tested (Fig. 1). In our experience, adenosine is more potent in suppressing population spikes in the dentate than in CA1. Accordingly, this difference in sensitivity to pertussis toxin may reflect variation in diffusion from the injection site or conceivably differences in adenosine receptor–G-protein coupling between these regions.

To confirm that treatment with pertussis toxin does affect G-proteins in the hippocampus, we assessed the ADP ribosylation of G-proteins biochemically. G-proteins ADP-ribosylated in vivo would be expected to exhibit reduced labelling with radiolabelled ADP-ribose in vitro. Accordingly, we compared in vitro labelling of hippocampal slice homogenates with [^{32}P]NAD in the presence of pertussis toxin. In slices from untreated animals, two bands were labelled with molecular weights of 41 and 39 kDa, that correspond to species of G_i and G_o, respectively. In slices from animals treated with pertussis toxin that were clearly resistant to adenosine, there was a large reduction in labelling of these bands in vitro (Fig. 4) providing evidence that pertussis toxin treatments in vivo have the intended effect.

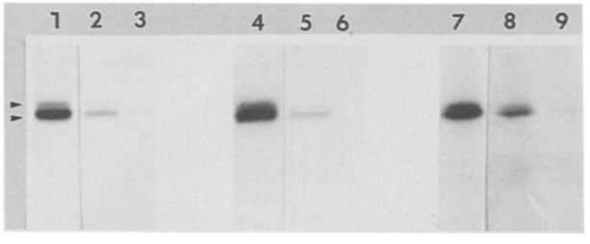

Fig. 4. Autoradiograms of the effect of intrahippocampal pertussis toxin injection on in vitro ADP-ribosylation of G proteins. The autoradiograms are of 3 separate experiments. Lanes 1, 4, and 7 shows in vitro ribosylation by pertussis toxin of two bands corresponding to G_i and G_o in hippocampal slices from control rats. Lanes 2, 5, and 8 demonstrate the greatly decreased in vitro ribosylation of both bands following pertussis toxin treatment in vivo. Lanes 3, 6, and 9 are from rats injected with pertussis toxin in vivo but not incubated with pertussis toxin in vitro, demonstrating the labelling occurs only in the presence of pertussis toxin.

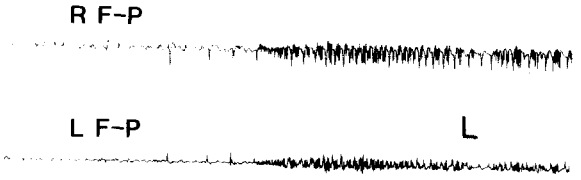

Fig. 5. Electroencephalographic seizure activity in a pertussis toxin-treated rat. EEG recordings of bipolar derivation between frontal and parietal (F–P) cortical screw electrodes from a rat 6 days after injection with 2 μg pertussis toxin into the right hippocampus. Tracings show normal activity followed by bilateral, high amplitude seizure activity. This rat displayed intermittent EEG seizure activity accompanied by running fits, vocalizations, wet dog shakes, and tonic-clonic convulsions. Calibration bars are 1 mV vertical, 1 s horizontal.

During the course of these treatments, we noted that animals often displayed intermittent abnormal behavior including wet dog shakes, suggestive of seizure activity. To determine whether these behaviors reflected seizure discharge, we monitored rats by chronic EEG recording techniques and found that electrical seizure activity was present intermittently in pertussis toxin treated rats beginning 2–3 days following treatment (Fig. 5). Presumably, this activity reflects blockade of inhibitory transmitters acting at G_i-linked receptors such as adenosine, γ-aminobutyric acid (GABA) and serotonin. However, to examine the alternative possibility that blockade of adenosine is secondary to the seizure activity, we subjected rats to maximal electroconvulsive seizures twice daily and prepared slices 10 min after the 7th seizure. In 3 slices from two rats adenosine (40 μM) reduced the CA1 population spike to less than 10% of the control response, indicating that repetitive seizure activity is not sufficient to block adenosine responsiveness.

Although adenosine's hyperpolarization of CA1 pyramidal neurons can be ascribed to activation of potassium channels, the basis of its presynaptic actions remains obscure. Our results provide evi-

dence for the involvement of a pertussis toxin-sensitive G-protein in this process. These findings fit well with a previous report that adenosine inhibition of glutamate release from cultured cerebellar granule cells is pertussis toxin sensitive[5]. In contrast to our results using intrahippocampal injections, intraventricular injection of pertussis toxin blocks the postsynaptic but not the presynaptic effects of adenosine[8]. Accordingly, the higher concentrations of pertussis toxin achieved with local injections of pertussis toxin[13] appear to be required to block presynaptic actions of adenosine. Similar resistance of presynaptic but not postsynaptic actions of baclofen to intraventricular injection of pertussis toxin has been reported[9], whereas local injection of pertussis toxin also blocks baclofen's presynaptic action (unpublished observations).

Phorbol esters, activators of protein kinase C (PKC), mimic the ability of pertussis toxin to block several inhibitory transmitters in the hippocampus[2,29]. These findings have prompted the hypothesis that PKC may exert these effects on many transmitters by phosphorylating and inactivating the common G-proteins involved as has been demonstrated in platelets[14]. In previous studies, we have demonstrated that phorbol esters block the ability of adenosine to suppress synaptic responses[27,29]. In this study, we have found that this action of adenosine is also blocked by pertussis toxin, suggesting that a similar action of PKC to phosphorylate and inactivate G-proteins may account for its blockade of adenosine.

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