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Cholinergic phosphatidylinositol modulation of inhibitory, G protein-linked, neurotransmitter actions: Electrophysiological studies in rat hippocampus

(protein kinase C/phorbol esters/baclofen/adenosine/muscarinic receptors)

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ABSTRACT **In electrophysiological studies using the rat hippocampal slice preparation, cholinergic agonists and phorbol 12,13-diacetate, a stimulator of protein kinase C, block the inhibitory actions of baclofen, a γ-aminobutyric acid B receptor agonist, and adenosine. Relative potencies of cholinergic agonists in stimulating the phosphatidylinositol system, as measured biochemically, parallel their activity in blocking adenosine assessed electrophysiologically. Electrical stimulation of cholinergic afferents also reverses adenosine's inhibitory action. These findings indicate that stimulation of protein kinase C by the phosphatidylinositol system mediates cholinergic blockade of adenosine and baclofen. As these inhibitory agonists act by way of receptors linked to GTP-binding proteins, protein kinase C's inactivation of the GTP-binding protein involved may account for this cholinergic action.**

Several lines of evidence indicate a prominent role for the phosphatidylinositol (PtdIns) cycle in mediating transmitter actions in the brain. Protein kinase C (PKC) and inositol trisphosphate receptors localized by autoradiographic techniques occur in very high concentrations in synaptic areas of the brain (1, 2). Phorbol esters that stimulate PKC (3) exert selective actions on ionic conductances in brain neurons (4–7). Direct injections of inositol trisphosphate into dorsal raphe neurons mimic transmitter actions thought to be linked to the PtdIns system (7).

Recent studies suggest that PKC can block inhibitory transmitter actions associated with GTP-binding regulatory proteins (G proteins). Thus, in platelets, phorbol esters antagonize the ability of epinephrine to inhibit adenylate cyclase (8). In oocytes, phorbol esters block the increased potassium conductance elicited by adenosine (9). In brain slices, phorbol esters diminish the ability of adenosine to inhibit adenylate cyclase (10). In previous electrophysiologic studies of hippocampal CA1 pyramidal neurons, we found that the synaptically activated late hyperpolarization (11) is blocked by phorbol esters (4). It has been suggested that the late hyperpolarization is mediated by γ-aminobutyric acid (GABA) acting at the $GABA_B$ receptor (12, 13), although transmitters such as adenosine (14, 15) and serotonin (16, 17) have similar effects and are also thought to act by way of a G protein. Furthermore, phorbol esters block the hyperpolarization caused by baclofen stimulation of $GABA_B$ receptors (18, 19). These interactions might reflect the ability of PKC to phosphorylate and thus inactivate the G proteins involved in mediating inhibitory transmitter actions (20). In the present study, we provide direct evidence that muscarinic cholinergic synaptic transmission, acting by way of the PtdIns cycle and PKC, blocks inhibitory effects of neurotransmitters mediated by receptor-regulated G proteins.

METHODS

Electrophysiological Recordings from Hippocampal Slices. Slices were obtained from adult male Sprague–Dawley rats by using techniques that have been described in detail elsewhere (21, 22). For some field potential recordings, slices were obtained from aged Fisher 344 rats (30 months, National Institutes of Health). One 400-μm-thick slice was held submerged in the recording chamber at 30°C. Temperature was regulated by a heating-cooling module (Cambion, Cambridge, MA) and was monitored within 1 mm of a slice by a hypodermic thermistor probe. Other slices were maintained in an incubation chamber at room temperature. The standard physiological saline was saturated with 95% O_2/5% CO_2 and consisted of (in mM) NaCl, 122.6; KCl, 3.5; $CaCl_2$, 2.5; $MgSO_4$, 2.0; NaH_2PO_4, 1.2; $NaHCO_3$, 26.2; and glucose, 10. In some experiments $CaCl_2$ and $MgSO_4$ were replaced by 2.0 $MnCl_2$ and 3.5 $MgCl_2$ to block synaptic transmission. The recording chamber provides constant perfusion and allows switching between salines by means of a valve.

Field potential recordings were made from the CA1 pyramidal cell layer with fiber-filled glass microelectrodes that contained 2 M NaCl and had impedances of 5–15 MΩ at 135 Hz. Field potentials were elicited routinely at 0.1 Hz by 50-μsec pulses from a bipolar stimulating electrode located in stratum radiatum near the junction of CA1 and CA3. Stimulation voltage was adjusted to a level just below that producing maximal population spike amplitude. Data were collected on a Nicolet 2090 digital oscilloscope and recorded on a Gould 60000 X–Y recorder.

Intracellular recordings were made from over 50 CA1 pyramidal neurons. Resting potentials varied from −55 to −73 mV and input resistances varied from 40 to 100 MΩ. Tetrodotoxin, 0.3 μM, was used to block sodium-dependent action potentials. During intracellular recordings, adenosine was ordinarily ejected from an independently positioned broken pipette (tip diameter ≈10 μm) containing 50 mM adenosine in 125 mM NaCl (pH 4.0). Ejection currents of 90–250 nA were typically used to avoid loss of the intracellular impalement by moving the iontophoretic pipette too close to the recording electrode. In some experiments adenosine, 100 μM, was applied by way of bath perfusion at neutral pH. Data were stored on FM tape and replayed onto a chart recorder for illustration.

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Abbreviations: PtdIns, phosphatidylinositol; PKC, protein kinase C; PAc_2, phorbol 12,13-diacetate; G protein, GTP-binding regulatory protein; GABA, γ-aminobutyric acid.

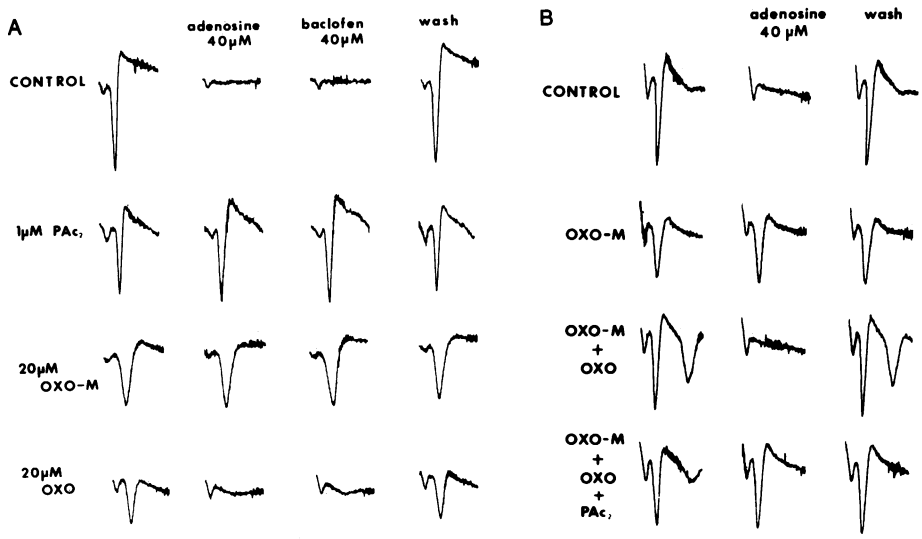

FIG. 1. Blockade of inhibitory actions of adenosine and baclofen by PAc_2 and oxotremorine-M. (*A*) Adenosine and baclofen completely inhibit the population spike recorded in CA1 stratum pyramidale. PAc_2, 1 μM which activates PKC, completely blocks their inhibitory action. This action is also produced by 20 μM oxotremorine-M (OXO-M), a full agonist at PtdIns turnover, but not by oxotremorine (OXO), a partial agonist. Tracings from each drug treatment were taken from separate experiments. (*B*) Adenosine suppression of the population spike is blocked by oxotremorine-M. Oxotremorine, which acts as a weak partial agonist at PtdIns turnover, reverses oxotremorine-M's blockade of adenosine. Stimulation of PKC by PAc_2 mimics oxotremorine-M's action. Tracings shown in *B* were taken sequentially during a single experiment.

Oxotremorine-M and oxotremorine-2 were a generous gift from S. K. Fisher (Ann Arbor, MI). Phorbol 12,13-diacetate (PAc_2) was obtained from LC Services (Woburn, MA). Other drugs were obtained from standard commercial sources.

RESULTS

Extracellular Recordings. In field potential recordings from the CA1 cell layer of rat hippocampal slices, the population spike response is reversibly abolished by adenosine (23, 24) and baclofen (25–27). The water-soluble phorbol ester PAc_2 at 1 μM fails to influence the population spike but abolishes the inhibitory effects of adenosine and baclofen (Fig. 1*A*). Fisher and collaborators have differentiated muscarinic cholinergic agonists on the basis of their efficacy in stimulating PtdIns turnover (28–30). In these biochemical studies, oxotremorine-M is a full agonist, whereas oxotremorine itself is only a weak partial agonist. In our electrophysiological experiments, oxotremorine-M, like PAc_2, abolishes the inhibitory effects of adenosine and baclofen. By contrast, oxotremorine has no influence on the actions of adenosine and baclofen. This effect of oxotremorine-M involves muscarinic receptors since it is fully antagonized by 50 nM atropine (data not shown). The ability of oxotremorine-M, but not oxotremorine, to mimic the effects of the phorbol ester suggests that the muscarinic cholinergic antagonism of adenosine and baclofen involve the PtdIns cycle. Since oxotremorine is an antagonist of muscarinic stimulation of PtdIns turnover (28), it should block this electrophysiological effect of oxotremorine-M. Indeed, we find a complete antagonism by oxotremorine of the ability of oxotremorine-M to reverse adenosine inhibition (Fig. 1*B*). As expected, addition of PAc_2 overcomes this effect of oxotremorine, since it directly stimulates PKC, effectively bypassing oxotremorine blockade of the muscarinic receptor.

Table 1. Cholinergic blockade of adenosine in rat hippocampus: Pharmacology of muscarinic agents

Active agonist	Concentration, μM	Inactive agonist	Concentration, μM
Oxotremorine-M	10	Oxotremorine	100
Carbachol	20	Acrecoline	100
Acetylcholine + 2 μM eserine	40	McN-A-343	100
Oxotremorine-2	50	Pilocarpine*	100

Values shown for active cholinergic agonists are the minimum concentrations of bath-applied agonists necessary to completely block the action of 40 μM adenosine or baclofen. At 100 μM the inactive agonists do not block 40 μM adenosine but completely antagonize the "active" effect of 20 μM oxotremorine-M. Cholinergics were applied 20 min prior to addition of adenosine or baclofen. Compounds that had no effect on the efficacy of adenosine or baclofen included 100 μM phenylephrine, 20 μM forskolin, 200 μM L-norepinephrine, 1 μM isoproterenol, and 200 μM serotonin.

*Pilocarpine was the only drug of this group that partially blocked adenosine. At 100 μM, it produced a 30% block of 40 μM adenosine and also prevented any further action of oxotremorine-M.

To further establish that the actions of oxotremorine-M are selectively mediated by those muscarinic receptors that act by way of the PtdIns cycle, we explored the effects of a series of cholinergic agonists that vary in their influences on the PtdIns cycle (Table 1). Acetylcholine, carbachol, and oxotremorine-2, which stimulate the PtdIns system, mimic the effects of oxotremorine-M, whereas arecoline and McN-A-343, which only weakly stimulate PtdIns turnover, fail to antagonize adenosine. Since these latter three agents have been shown to be antagonists of muscarinic-induced PtdIns turnover, we evaluated their influences on oxotremorine-M's effect and have found that they also block its action.

We sought to establish whether these drug effects reflect cholinergic synaptic transmission. Accordingly, we assessed the effects of stimulation in the vicinity of cholinergic axons and terminals of the septo-hippocampal pathway on adenosine's inhibitory action (Fig. 2). Like administration of oxotremorine-M and PAc_2, stimulation of cholinergic fibers reverses the inhibitory actions of adenosine with a slow time course resembling that of the synaptic effects of the septo-hippocampal cholinergic pathway (31, 32). The involvement of muscarinic cholinergic neurotransmission in this stimula-

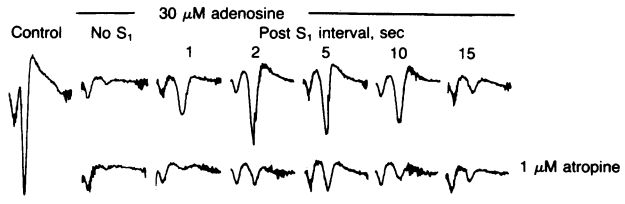

FIG. 2. Stimulation of cholinergic afferents blocks adenosine. Stimulation in stratum radiatum elicits a population spike that is suppressed by adenosine, 30 μM (no S_1). Stimulation in the vicinity of cholinergic afferents to CA1 (S_1; 40 Hz/0.5 sec) in the presence of eserine (2 μM), an acetylcholinesterase inhibitor, reversibly blocks adenosine's inhibition for 10–15 sec following S_1. Atropine (1 μM) blocks the effects of cholinergic stimulation.

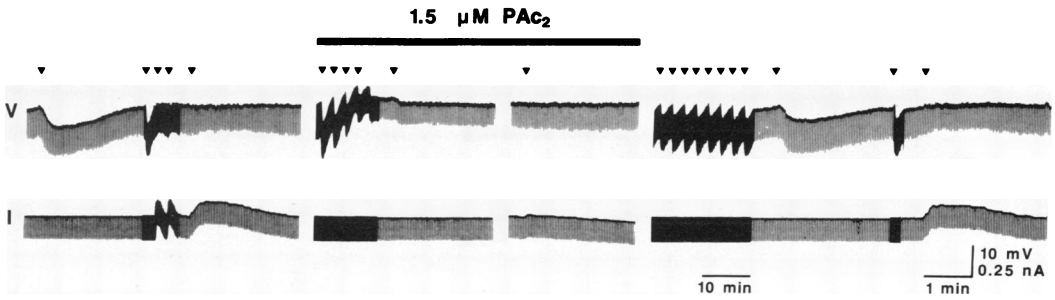

FIG. 3. Phorbol ester blocks adenosine-induced outward current. Bath application of PAc_2 completely blocks intracellularly recorded response to iontophoretically applied adenosine (10 sec/250 nA per 2.5-min intervals; triangles). Slow variations in voltage (V, top row) indicate current clamp recording, whereas slow variations in current (I, bottom row) indicate manual voltage-clamp recording. After control responses, PAc_2 (1.5 μM) was applied for 26 min (dark bar). Voltage and current responses were blocked by PAc_2, which was then washed for 78 min before the recovered responses were recorded. Fast downward deflections here and in Fig. 4 are due to injection of 100-msec hyperpolarizing constant current pulses. Bridge balance was constantly monitored and adjusted when necessary.

tion is confirmed by the antagonism of these effects by atropine.

Blunting of cholinergic effects on hippocampal CA1 neurons has been reported in aged rats (33). Accordingly, we evaluated muscarinic responses in slices from 30-month-old Fisher 344 rats. The potency and pharmacologic profile of muscarinic agonists (oxotremorine-M, $n = 3$; carbachol, $n = 2$; oxotremorine, $n = 3$) in blocking adenosine's actions is unaltered in slices from these aged rats.

Intracellular Recordings. Intracellular recordings from CA1 hippocampal pyramidal cells directly demonstrate hyperpolarization by adenosine (14, 15) and its antagonism by PAc_2 in a reversible fashion (Fig. 3). Under manual voltage clamp, the outward current elicited by adenosine is antagonized reversibly by PAc_2.

We wondered whether the interactions of muscarinic stimulation and adenosine are merely secondary to muscarinic effects on membrane potential and resistance rather than reflecting biochemical interactions at a second messenger level. Accordingly, we compared the effects of carbachol and oxotremorine upon the current responses to adenosine while the membrane potential was clamped at the control level (Fig. 4). Strikingly, though carbachol and oxotremorine depolarize the membrane and increase input resistance to the same extent (Table 2), carbachol is substantially more effective than oxotremorine in blocking the adenosine-elicited outward current. Reduction in the adenosine response is readily reversible (Fig. 4*A*) and the differences between carbachol and oxotremorine are apparent at 20 μM and 50 μM concentrations (Fig. 4 *B* and *C*).

Conceivably, effective muscarinic agonists could act indirectly by releasing some substance from nerve terminals that would in turn antagonize adenosine. To rule out this possibility, we performed experiments in a low Ca/Mn-containing saline that abolishes synaptic transmission. Carbachol ($n = 3$) continues to block adenosine under these conditions. To control for possible direct effects of iontophoretic ejection current and pH, we also bath-applied adenosine at neutral pH and found that the actions of PAc_2 ($n = 2$) and of muscarinic agonists ($n = 9$) are unaffected.

DISCUSSION

The main finding of our study is that muscarinic cholinergic stimulation, by way of the PtdIns cycle, blocks effects of inhibitory transmitters mediated by receptor-regulated G proteins. Several experimental findings support this conclusion. The effects of muscarinic agonist application or cholinergic pathway stimulation mimic those of phorbol esters. Among muscarinic drugs, only those known to stimulate the PtdIns cycle block adenosine's inhibition. Since our extracellular field potential recordings reveal antagonism by muscarinic stimulation of adenosine and $GABA_B$ receptor-mediated responses, the cholinergic response is not restricted to one transmitter but can be generalized to inhibitory responses involving receptors linked to G proteins. In intracellular recordings, phorbol esters also antagonize the effects of serotonin and baclofen as well as adenosine (refs. 11 and 19; unpublished observations). The antagonism by pertussis toxin of inhibitory actions of baclofen and serotonin further supports the role of a G protein in their effects (19).

Our intracellular recordings establish that blockade of adenosine by muscarinic stimulation cannot be accounted for simply by muscarinic effects on membrane potential and resistance. Indeed, these experiments clearly demonstrate that the membrane potential and resistance changes are elicited similarly by carbachol and oxotremorine, whereas the two drugs differ markedly in their interactions with adenosine. A similar pharmacological approach has identified other muscarinic actions that may be mediated by way of the PtdIns cycle. Oxotremorine-M causes a slow excitation of cerebral cortical pyramidal neurons not manifest with oxotremorine (34). Similarly, in CA1 pyramidal neurons *in vivo*, excitatory responses to muscarinic agents that strongly stimulate the PtdIns cycle undergo rapid desensitization (35).

We propose that in the hippocampus, muscarinic stimulation influences the actions of inhibitory neurotransmitters that work by way of G proteins by inactivating the G proteins themselves (Fig. 5). PKC phosphorylates G_i, the inhibitory G protein of adenylate cyclase, and thereby inactivates its function in platelets (8, 20). Of course, phosphorylation of an associated ion channel could also explain our findings but such effects have not been demonstrated.

Since many neurotransmitters act through the PtdIns cycle as well as through G protein-regulated adenylate cyclase systems and/or G protein-linked ion channels, the "cross talk" at the level of second messengers that we propose may have widespread significance. This model may account for numerous reports of synaptic interactions between different neurotransmitters. For instance, phorbol esters and neurotransmitters that act through the PtdIns cycle enhance receptor-mediated elevation of cyclic AMP levels (36–38). These effects could result from phosphorylation by PKC of G_i, which would diminish the inhibition of adenylate cyclase by endogenous transmitters, thus amplifying the apparent stimulation by applied transmitters. Magistretti and Schordoret (39) have similarly shown that stimulation of α_1-adrenergic and H_1-histamine receptors that activate the PtdIns cycle enhances the ability of vasoactive intestinal peptide to stimulate cyclic AMP accumulation, which could also involve a similar mechanism. In this case, the noradrenergic innervation to the cortex is orthogonal to the vaso-

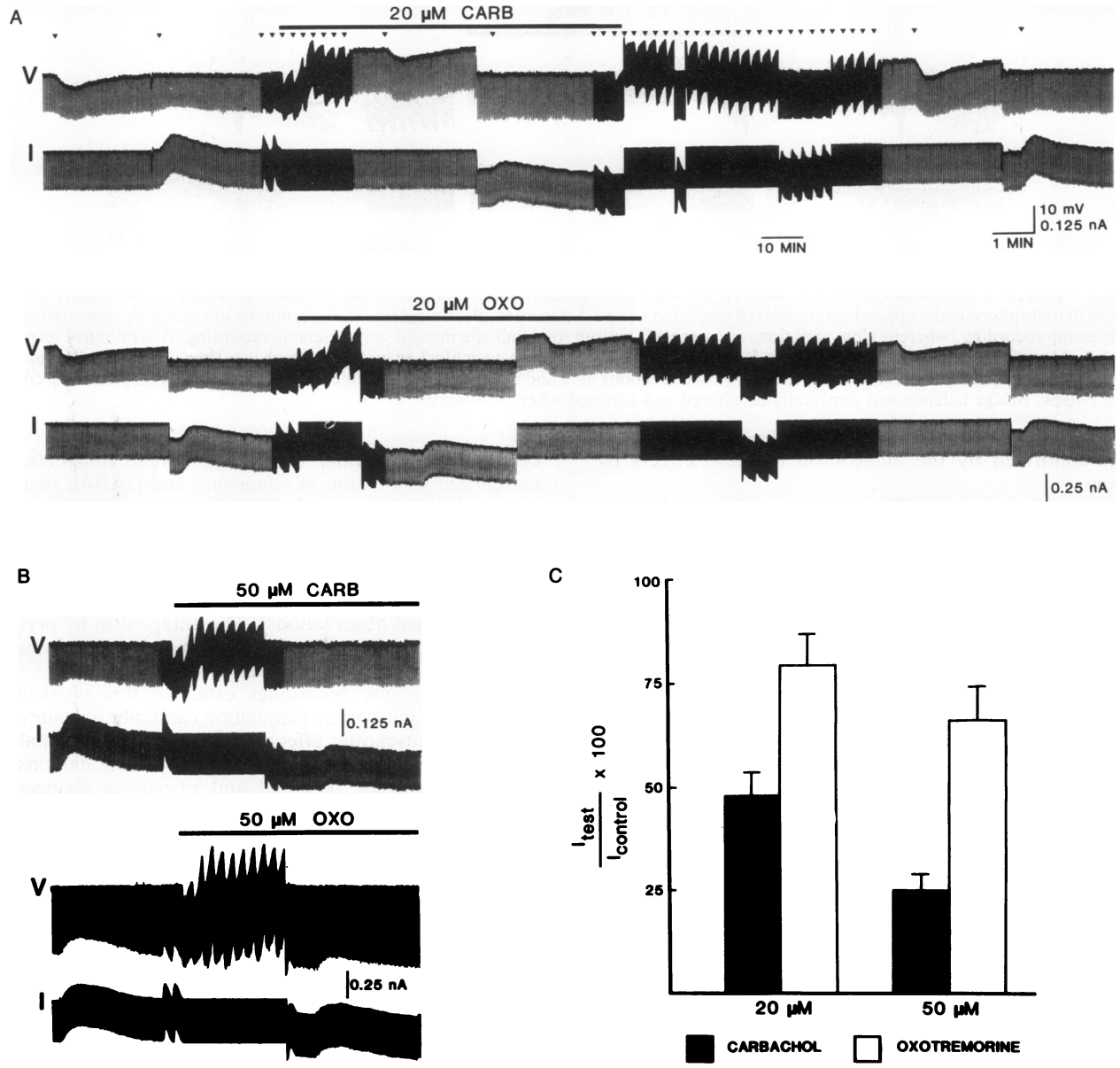

FIG. 4. Carbachol blocks adenosine responses more effectively than oxotremorine. Each row represents the continuous recording from a single cell. Traces are interpreted as indicated in the legend to Fig. 3. Carbachol (CARB) and oxotremorine (OXO) cause comparable membrane depolarization and increase in input resistance, but carbachol depresses the adenosine response to a greater extent. (*C*) Group data of the effects of the muscarinic agonists on the peak of the adenosine-induced current.

active intestinal peptide cortical interneurons (40, 41); however, a similar interaction between second messenger systems may also contribute to the marked interactions described among cotransmitters released from a single neuron.

Table 2. Comparison of effects of oxotremorine and carbachol on CA1 pyramidal cell properties

	Concentration, μM	*n*	Depolarization, mV	% increase in input resistance	% decrease in adenosine-elicited outward current
Carbachol	20	4	9.8 ± 2.14	10.8 ± 8.22	52.5 ± 5.74
Oxotremorine	20	5	8.6 ± 3.60	11.5 ± 3.94	20.6 ± 7.50
t (df = 7)			0.379	−0.182	6.987
Significance			NS	NS	$P < 0.002$
Carbachol	50	4	7.7 ± 4.52	22.0 ± 12.02	75.0 ± 3.56
Oxotremorine	50	6	8.2 ± 2.98	18.5 ± 4.76	34.2 ± 7.78
t (df = 9)			−0.205	0.659	9.566
Significance			NS	NS	$P < 0.002$

Data are expressed as mean ± SD. NS, not significant.

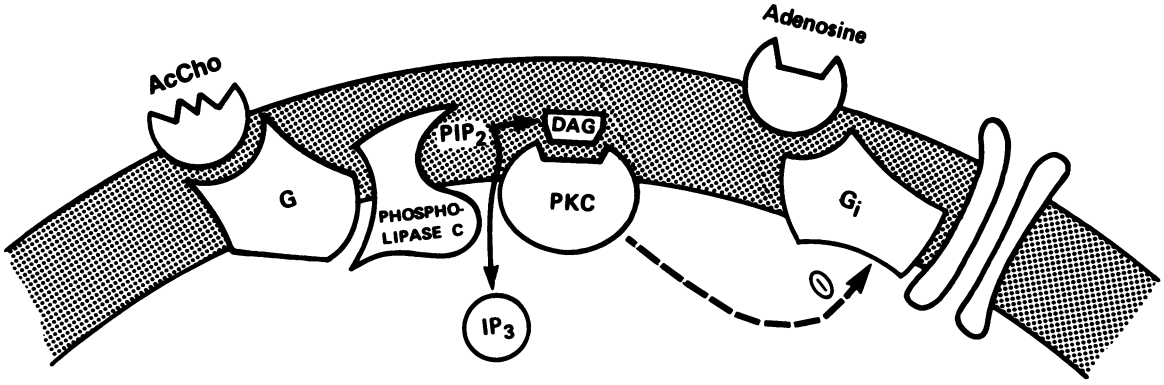

FIG. 5. Schematic model of interaction between PtdIns system and inhibitory responses mediated by G proteins. Stimulation of phospholipase C by acetylcholine (AcCho) at muscarinic receptors is mediated by an as yet unidentified G protein. Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) generates diacylglycerol (DAG) and inositol trisphosphate (IP_3). Stimulation of PKC by DAG results in the inactivation of the G protein coupling the adenosine receptor to ion channels, thought to be G_i, or a closely related G protein.

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