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Phencyclidine is a negative allosteric modulator of signal transduction at two subclasses of excitatory amino acid receptors

(glutamate receptors/phosphatidylinositol/calcium uptake/cyclic GMP/granule cells)

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ABSTRACT **Phencyclidine (PCP) and some of its pharmacological congeners inhibit the signal transduction at specific excitatory amino acid receptors of cerebellar granule cells in primary cultures. These drugs do not bind to the transmitter recognition sites, and affinity of this specific binding site is increased by the presence of the transmitter bound to its recognition sites. PCP inhibits phosphatidylinositol phosphate hydrolysis mediated by Mg^{2+}-sensitive glutamate receptors (G_{P1}) but not that mediated by Mg^{2+}-insensitive glutamate receptors (G_{P2}). In addition, PCP inhibits Ca^{2+} influx and cGMP formation mediated by the activation of Mg^{2+}-sensitive glutamate receptors (G_{C1}) but not that mediated by Mg^{2+}-insensitive glutamate receptors (G_{C2}). In this cell culture the activation of phosphatidylinositol phosphate hydrolysis by muscarinic receptor agonists is not affected by PCP. Since PCP inhibits noncompetitively G_{P1} and G_{C1} signal transduction it may act as a negative allosteric modulator of signal transduction at both receptors. The pharmacological profile of PCP and its congeners delimits a class of drugs modulating allosterically the action of the primary transmitter at G_{P1} and G_{C1} receptors. These drugs need the presence of the transmitter to act and they cannot be termed inverse agonists because they are devoid of activity in the absence of the transmitter; moreover, they do not bind to the transmitter recognition site nor do they prevent the transmitter binding to its recognition sites.**

In primary cultures of cerebellar granule cells, excitatory amino acid recognition sites are coupled to specific signal-transducing mechanisms. These mechanisms include (*i*) the hydrolysis of membrane phosphatidylinositol phosphates (1, 2) and (*ii*) the activation of receptor-operated Ca^{2+} influx, leading to an enhanced intracellular Ca^{2+} content (3, 4) and a consequent increase in cGMP formation (5). Hence, we have proposed a classification of excitatory amino acid receptors that takes into account the different modalities of receptor operation. We do not know yet whether recognition sites for transmitters are located in the signal-transducing molecule, but at least in the case of those recognition sites coupled to the transducer by a GTP-binding protein, it appears that the transmitter recognition site is located in a molecule different from that operating the signal transduction. Our classification distinguishes between excitatory amino acid recognition sites coupled with phosphatidylinositol phosphate hydrolysis (G_P) and those coupled with the activation of Ca^{2+} influx (G_C) (6). G_{P1} and G_{C1} receptors are activated by glutamate and aspartate and are antagonized by 2-amino-5-phosphonovalerate (APV) and inhibited by Mg^{2+}. The G_{P2} receptors are activated by glutamate and are insensitive to Mg^{2+} and APV. The two classes of G_P receptors can also be differentiated because they have a different sensitivity to synthetic agonists. The G_{P1} receptor is activated by *N*-methyl-D-aspartate and the G_{P2} receptor is activated by quisqualate.

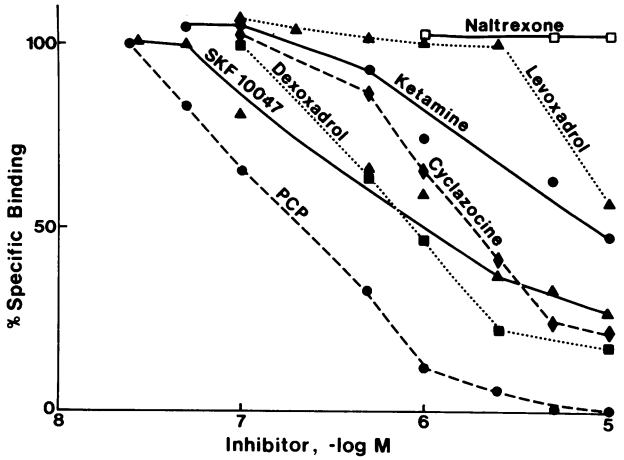

FIG. 1. Inhibition of specific [^{3}H]PCP binding by pharmacological congeners of PCP in membranes prepared from cultured cerebellar granule cells. Each point is the mean value from a representative experiment performed in triplicate and repeated at least two times with similar results.

The two classes of G_C receptors are also differentiated by their activation by synthetic agonists *N*-methyl-D-aspartate (G_{C1}) and kainate (G_{C2}). The G_{C2} receptor is preferentially antagonized by *cis*-2,3-piperidinedicarboxylate and is insensitive to Mg^{2+} inhibition. Although the endogenous agonist of G_{C2} is still unknown, it is considered a glutamate receptor because of the structural similarity between kainate and glutamate. Since another class of amino acid receptors, which is activated by the endogenous inhibitory ligand γ-aminobutyric acid (GABA), is modulated by allosteric sites to which two types of drugs bind with high affinity and increase (benzodiazepines) or decrease (β-carbolines) the probability that GABA opens receptor-operated Cl^- channels, we wondered whether a similar modulation was shared by signal transduction at G_{P1} and G_{C1} receptors. Phencyclidine (PCP) and a number of its pharmacological congeners act as negative allosteric modulators of signal transduction at G_{P1} and G_{C1} receptors of cerebellar granule cells in primary culture. In addition, as in the case of the GABA receptor, the affinity of the allosteric modulatory sites for PCP is increased when specific G_{P1} and G_{C1} agonists are bound to their receptors.

MATERIALS AND METHODS

Granule Cell Cultures. Primary cultures of cerebellar granule cells were prepared from 8-day-old Sprague–Dawley

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Abbreviations: PCP, phencyclidine; GABA, γ-aminobutyric acid; APV, 2-amino-5-phosphonovalerate; [^{3}H]Ins-1-*P*, [^{3}H]inositol 1-monophosphate.

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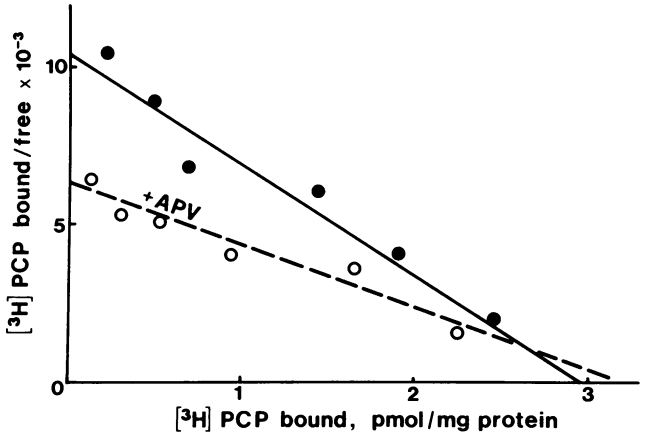

FIG. 2. Scatchard plot of [^{3}H]PCP binding in membranes prepared from cultured cerebellar granule cells in the absence (●) and presence (○) of 1 mM APV.

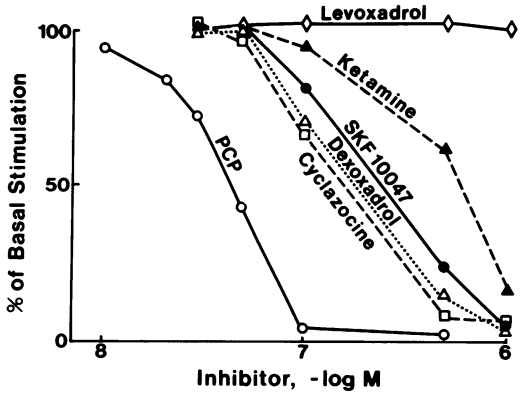

FIG. 3. Dose-dependent inhibition of G_{P_I} receptor-coupled signal transduction by PCP and its functional analogues in cultured cerebellar granule cells. The inhibition is expressed as the percentage of the basal formation of [^{3}H]Ins-1-*P* in the absence of extracellular Mg^{2+}. Each point is the mean of at least four experiments.

rats (Zivic–Miller, Pittsburgh) as described (1), according to the method of Gallo *et al.* (7). These cultures contain >90% of granule neurones, about 5% of GABA-ergic interneurones, and a small amount of astrocytes, oligodendrocytes, and endothelial cells as contaminants (1, 8). Cultures at 7–9 days *in vitro* were used in all of the experiments.

Measurement of PCP Binding. Granule cells were harvested, homogenized, and centrifuged twice for 20 min at 45,000 × *g* in 10 mM Tris·HCl buffer (pH 7.4). The final pellet was resuspended in the same buffer to have a protein concentration of 1.5–2 mg/ml. Incubations included 100 μl of membranes and a range of concentrations of [^{3}H]PCP in a final volume of 0.5 ml. Nonspecific binding was determined with 20 μM PCP. Incubation was terminated by filtration through Whatman GF/C glass filters presoaked in 0.05% polyethylenimine; this was followed by three washes with 5 ml of ice-cold buffer.

Biochemical Measurements. The uptake of $^{45}Ca^{2+}$ was measured in cultures of cerebellar granule cells, as described (3). Phosphatidylinositol phosphate hydrolysis was estimated by measuring the accumulation of [^{3}H]inositol 1-monophosphate ([^{3}H]Ins-1-*P*) in cerebellar granule cells, as described (1). All of the experiments were performed in the presence of 7 mM Li^+ to inhibit the conversion of [^{3}H]Ins-1-*P* into [^{3}H]inositol (9). Intracellular cGMP levels were estimated by RIA, as described (5).

Chemicals. [*piperidyl*-3,4-^{3}H]PCP (47 Ci/nmol; 1 Ci = 37 GBq), *myo*-[2-^{3}H]inositol (12 Ci/mmol), $^{45}CaCl_2$ (20 Ci/g), and ^{125}I-labeled cGMP RIA kit were obtained from New England Nuclear. PCP and PCP analogues were a generous gift from K. J. Kellar (Department of Pharmacology, Georgetown University School of Medicine). All other chemicals were purchased from Sigma.

RESULTS

Specific Binding of [^{3}H]PCP to Granule Cell Membranes. In membranes prepared from cerebellar granule cells, [^{3}H]PCP binds to a single class of saturable sites with an apparent K_d ranging from 170 to 290 nM and a B_{max} of about 4 pmol/mg of protein. The specifically bound [^{3}H]PCP was displaced by the pharmacological congeners of PCP with the following rank order of potency, PCP > SKF 10047 = dexoxadrol > cyclazocine > ketamine >> levoxadrol, and was not displaced by naltrexone (Fig. 1). The specific binding of [^{3}H]PCP was enhanced by the addition of glutamate (0.1 mM) to the membranes; however, this increase never exceeded 20% due to the endogenous glutamate already present in the membrane preparation. To assess the validity of such inferences, 1 mM APV was added to antagonize the action of the endogenous glutamate. In presence of APV the affinity

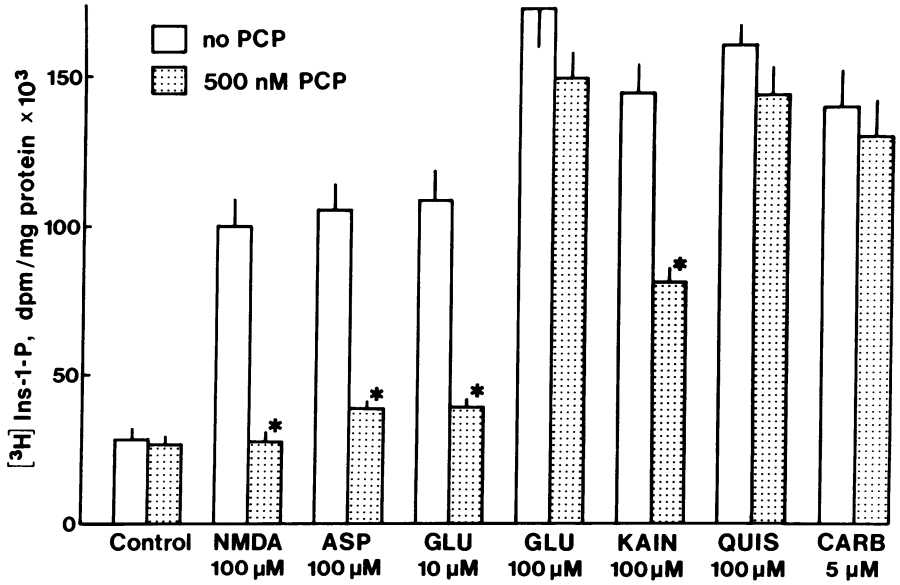

FIG. 4. PCP selectively inhibits signal transduction at G_{P_I} receptors in cultured cerebellar granule cells. The experiment was performed in Mg^{2+}-free conditions and in the presence of 5 μM APV to inhibit the action of endogenous excitatory amino acids. Values are means ± SEM of at least six experiments. *, $P < 0.01$, if compared with values obtained in the absence of PCP. NMDA, *N*-methyl-D-aspartate; ASP, aspartate; GLU, glutamate; KAIN, kainate; QUIS, quisqualate; CARB, carbamoylcholine.

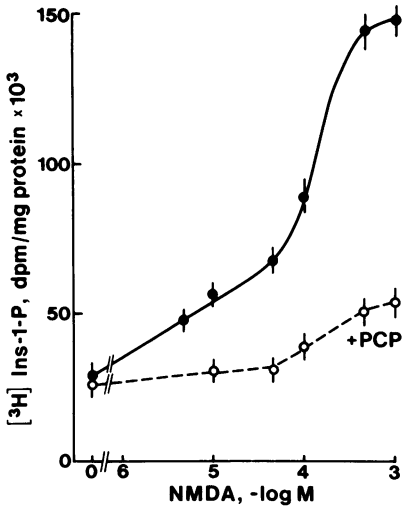

FIG. 5. Noncompetitive inhibition of signal transduction at the G_{P1} receptor by PCP. Phosphatidylinositol phosphate hydrolysis was stimulated by different concentrations of *N*-methyl-D-aspartate (NMDA) in Mg^{2+}-free conditions and in the presence of 5 μM APV to inhibit the action of the endogenous excitatory amino acids. PCP concentration = 0.5 μM. Values are means ± SEM of at least three experiments.

constant of [^{3}H]PCP binding almost doubled without changes in the B_{max} (Fig. 2).

Inhibition of Phosphatidylinositol Phosphate Hydrolysis by PCP. In granule cells, the basal phosphatidylinositol phosphate hydrolysis is greatly enhanced in the absence of Mg^{2+} ions, because these ions inhibit the activation of the G_{P1} receptor by endogenous glutamate (2). This accumulation of [^{3}H]Ins-1-*P* was inhibited by PCP and its pharmacological congeners in a concentration-dependent manner (Fig. 3). The EC_{50} for PCP was 50 nM and the order of potency of the active analogues was PCP > cyclazocine = dexoxadrol = SKF 10047 > ketamine. This rank order of potency was similar to that observed for the inhibition of [^{3}H]PCP specific binding (Fig. 1).

PCP inhibited the phosphatidylinositol phosphate hydrolysis elicited by specific ligands of G_{P1} receptors (Fig. 4) but not that elicited by agonists of G_{P2} or muscarinic receptors (Fig. 4). Thus, the stimulation of phosphatidylinositol phosphate hydrolysis produced by *N*-methyl-D-aspartate, aspartate, or low concentrations of glutamate was entirely abolished by 0.5 μM PCP, whereas that elicited by carbamoylcholine or quisqualate was unaffected. The activation of phosphatidylinositol phosphate hydrolysis caused by kainate is mediated to a large extent by an enhanced release of glutamate acting at the G_{P1} receptor (1); this explains why the stimulation of phosphatidylinositol phosphate hydrolysis by kainate was partially inhibited by PCP (Fig. 4) as well as by APV (1). PCP inhibited signal transduction at the G_{P1} receptor in a noncompetitive manner since even 1 mM *N*-methyl-D-aspartate could not overcome the action of 0.5 μM PCP (Fig. 5).

PCP Inhibition of G_{C1} Receptors. In primary cultures of cerebellar granule cells, glutamate, aspartate, and *N*-methyl-D-aspartate stimulated the influx of Ca^{2+} and the formation of cGMP in the absence of extracellular Mg^{2+} (4, 5). This stimulation was abolished by 1 μM PCP (Table 1). On the other hand, the stimulation of $^{45}Ca^{2+}$ uptake and cGMP formation mediated by way of the activation of the G_{C2} receptors by kainate was not reduced by PCP. PCP inhibited only the APV-sensitive component of the kainate effect (Table 2) mediated through the release of endogenous glutamate and subsequent activation of the G_{C1} receptor. As in the case of the G_{P1} receptor, the inhibition of signal transduction at the G_{C1} receptors by PCP was concentration-dependent (Fig. 6) and noncompetitive (Fig. 7).

DISCUSSION

PCP and its pharmacological congeners (benzomorphans and substituted dioxolanes) inhibit electrophysiological responses to *N*-methyl-D-aspartate, aspartate, and glutamate (11–18) but not to quisqualate and kainate (11–15). In rat striatum PCP can also suppress the *N*-methyl-D-aspartate-stimulated release of acetylcholine (19) and prevent the neurotoxic effects of *N*-methyl-D-aspartate but not those of kainate (20, 21). Autoradiographic studies using ^{3}H-labeled *N*-[1-(2-thienyl)cyclohexyl]piperidine to label PCP binding sites have shown that these sites and the *N*-methyl-D-aspartate-displaceable [^{3}H]glutamate binding sites have a similar distribution in the rat brain (22). In addition, glutamate enhances PCP binding to its specific recognition sites in brain membranes (10, 23). From these reports and from our studies it can be suggested that in neuronal membranes the recognition sites for excitatory amino acids and those for PCP are not only contiguous but they also functionally interact. This interaction is reminiscent of the interactions between $GABA_A$ and β-carboline recognition sites.

In the present study we demonstrate that PCP acts as a negative allosteric modulator of signal transduction at two specific subclasses of excitatory amino acid receptors, G_{P1} and G_{C1}, we have characterized in primary cultures of cerebellar granule cells. Signal transduction of these two receptor subtypes is activated by glutamate, aspartate, and *N*-methyl-D-aspartate and antagonized by APV and by micromolar concentrations of Mg^{2+} (1–6). PCP fails to affect signal transduction at G_{P2} receptors (activated by quisqualate and high concentrations of glutamate) and at G_{C2} receptors (activated by kainate). In addition, PCP does not inhibit the stimulation of phosphatidylinositol phosphate hydrolysis mediated by the activation of muscarinic cholinergic receptors.

The PCP inhibition of signal transduction mediated by G_{P1} and G_{C1} receptors exhibits a noncompetitive kinetics. More-

Table 1. PCP inhibits the stimulation of $^{45}Ca^{2+}$ uptake and cGMP formation mediated by the activation of G_{C1} receptors in cultured cerebellar granule cells

	$^{45}Ca^{2+}$ uptake, nmol/mg of protein		cGMP formation, pmol/mg of protein	
Agonist, 50 μM	Without PCP	With PCP	Without PCP	With PCP
None	6.8 ± 1.3	2.9 ± 0.4*	3.5 ± 1.0	1.4 ± 0.7*
N-Methyl-D-aspartate	33 ± 4.6	8.6 ± 0.9*	38 ± 3.0	3.5 ± 1.5*
Aspartate	51 ± 4.1	5.6 ± 1.1*	27 ± 8.1	1.6 ± 0.4*
Glutamate	64 ± 7.3	11 ± 2.4*	35 ± 4.2	1.4 ± 0.6*

The concentration of PCP was 1 μM. The experiment was performed in Mg^{2+}-free conditions. Values are means ± SEM from at least nine determinations.
*$P < 0.001$ as compared with values without PCP.

Table 2. PCP fails to inhibit signal transduction at the G_{C2} receptors in cultured cerebellar granule cells

	$^{45}Ca^{2+}$ uptake, nmol/mg of protein		cGMP formation, pmol/mg of protein	
	Without PCP	With PCP	Without PCP	With PCP
Basal	3.8 ± 0.3	2.6 ± 0.2*	1.4 ± 0.2	1.0 ± 0.3
50 μM kainate	24 ± 3.7	12 ± 2.1*	17 ± 2.5	12 ± 0.7*
+ 100 μM APV	13 ± 2.2	10 ± 2.0	11 ± 0.9	11 ± 0.4

The concentration of PCP was 1 μM. The experiment was performed in the presence of 1 mM Mg^{2+}. The stimulation by kainate was tested in the presence of 100 μM APV to eliminate the stimulation of G_{Cl} receptor by endogenously released glutamate. APV alone did not affect the basal values. Values are means ± SEM from at least six determinations.
*$P < 0.01$ as compared with values without PCP.

over, PCP interacts with specific membrane recognition sites (24–27); though they are distinct from the excitatory amino acid recognition sites, the affinity of PCP binding sites is increased when glutamate receptors bind an agonist. These data taken together indicate that PCP is a negative allosteric modulation of G_{P1} and G_{Cl} receptors. Cerebellar granule cells in culture express a single population of specific and saturable PCP binding sites with an affinity oscillating according to the degree of glutamate receptor occupancy (K_d ranging from 170 to 290 nM). This affinity but not the B_{max} is reduced to 540 nM in the presence of the glutamate antagonist APV (Fig. 2). These results suggest that in the supramolecular structure of G_{P1} and G_{Cl} receptors, the recognition sites for excitatory amino acids and PCP interact in such a way that the binding of glutamate to its site increases the affinity of PCP binding site for its specific ligands.

The molecular mechanism whereby PCP and its pharmacological congeners inhibit signal transduction at G_{P1} and G_{Cl} receptors remains to be elucidated. The density or the affinity of [^{3}H]glutamate binding sites in G_{P1} and G_{Cl} receptors is unchanged by PCP; these drugs do not affect the displacement of [^{3}H]glutamate binding by aspartate in membranes prepared from primary cultures of cerebellar granule cells (data not shown). However, it must be stressed that in the membranes of these "glutamatergic" cells the glutamate contaminations are particularly relevant; therefore, the high-affinity glutamate receptors cannot be studied readily. A similar explanation applies to the observations of Monaghan and Cotman (28) that in rat brain synaptic membranes the *N*-methyl-D-aspartate-displaceable [^{3}H]glutamate binding cannot be displaced by ketamine and cyclazocine. An action of PCP on the cationic channel associated with G_{Cl} receptors has been proposed as a mechanism for this interaction (23). Such a mechanism could not explain the inhibition of signal transduction at G_{P1} receptors since the stimulation of phosphatidylinositol phosphate hydrolysis is dissociated from an enhanced influx of extracellular Ca^{2+} (6). It can be speculated that, in G_{P1} and G_{Cl} receptors, PCP action is allosterically mediated by a receptor protein phosphorylation.

In conclusion, in primary cultures of cerebellar granule cells, PCP and its pharmacological congeners act as negative allosteric modulators of signal transduction at G_{P1} and G_{Cl} excitatory amino acid receptors. As in the case of the $GABA_A$ receptor (29, 30), the domain for the allosteric modulation of G_{Cl} and G_{P1} receptors cannot be defined with precision at this time; however, the discovery of an endogenous ligand for PCP recognition sites (31) indicates that these sites may have a physiological role in modulating excitatory amino acid signal transduction at G_{P1} and G_{Cl} receptors.

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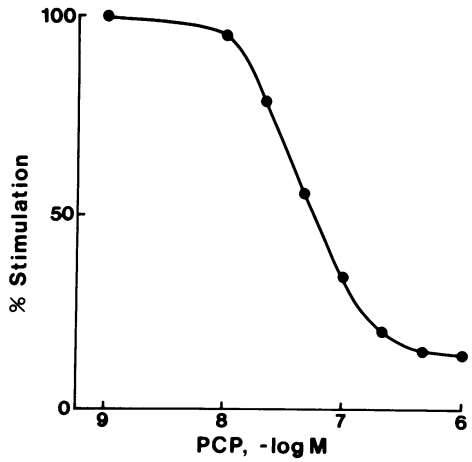

FIG. 6. Concentration-dependent inhibition of G_{Cl} receptor-coupled signal transduction in primary cultures of cerebellar granule cells. $^{45}Ca^{2+}$ uptake was stimulated by 50 μM *N*-methyl-D-aspartate in Mg^{2+}-free conditions. Values are expressed in percent of stimulation and represent means of at least three experiments.

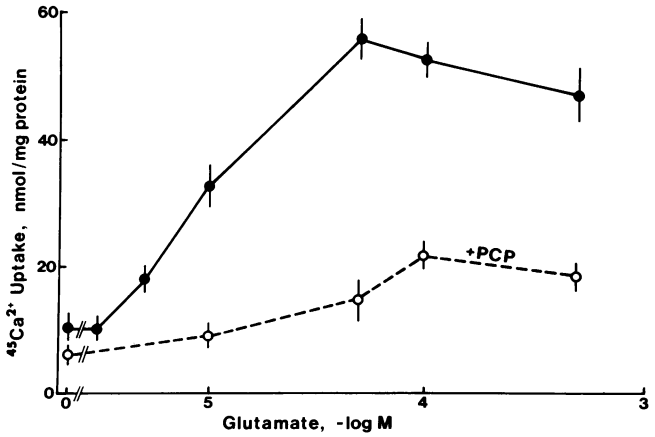

FIG. 7. Noncompetitive inhibition of signal transduction at the G_{Cl} receptor by PCP in cultured cerebellar granule cells. $^{45}Ca^{2+}$ uptake was stimulated by different concentrations of glutamate in Mg^{2+}-free conditions. Values are means ± SEM from at least four experiments.

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