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Metabotropic Glutamate Receptors Modulate [^{3}H]Acetylcholine Release From Cultured Amacrine-Like Neurons

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Retinal amacrine cells express metabotropic glutamate receptors (mGluRs), but their physiological role is unknown. We investigated the effect of mGluR on [^{3}H]acetylcholine release ([^{3}H]ACh) from cultured chick amacrine-like neurons. Activation of group III mGluR with the agonist L(+)-2-amino-4-phosphonobutyric acid (L-AP4) inhibited [^{3}H]ACh release evoked by 25 mM KCl in a dose-dependent manner, and this effect was sensitive to pertussis toxin. In contrast, activation of group I or II mGluR with (S)-3,5-dihydroxyphenylglycine (DHPG) and (2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV), respectively, did not affect significantly [^{3}H]ACh release. The effect of L-AP4 on [^{3}H]ACh release was sensitive to nitrendipine, suggesting that it is, at least in part, due to inhibition of L-type Ca^{2+} channels. Activation of group III mGluR also partly inhibited ω-conotoxin GVIA-sensitive Ca^{2+} channels, coupled to [^{3}H]ACh release. The L-AP4 did not affect the cAMP levels measured in amacrine-like neurons depolarized with 25 mM KCl or stimulated with forskolin, indicating that the effect of group III mGluR on [^{3}H]ACh release is not due to inhibition of adenylyl cyclase activity. Inhibition of protein kinase A with KT-5720 was without effect on [^{3}H]ACh release evoked by 25 mM KCl, further indicating that the effect of group III mGluR on [^{3}H]ACh release cannot be attributed to the inhibition of the kinase. The effect of L-AP4 on [^{3}H]ACh release was reversed by DHPG or by DCG-IV, and activation of group II mGluR also partially inhibited cAMP production stimulated by forskolin. Taken together, our results show that the effect of group III mGluR on [^{3}H]ACh release may be due to a direct inhibition of L- and N-type Ca^{2+} channels and is modulated by group I and group II mGluR. J. Neurosci. Res. 58:505–514, 1999. © 1999 Wiley-Liss, Inc.

Key words: L-AP4; calcium channels; cAMP; G proteins; retina

INTRODUCTION

In addition to activating ionotropic receptors, glutamate also acts on G protein-coupled receptors, modulating the production of intracellular second messengers. At least eight subtypes of metabotropic glutamate receptors (mGluR) have been revealed, and they were classified into three distinct groups based on sequence homology, pharmacology, and coupling to intracellular signalling mechanisms (Conn and Pin, 1997). The first group consists of subtypes mGluR1 and mGluR5, which activate phospholipase C, whereas members of the second (mGluR2 and mGluR3) and third (mGluR4, mGluR6, mGluR7, and mGluR8) groups are negatively coupled to adenylate cyclase. mGluR6 is coupled to the activation of cGMP phosphodiesterase in ON-bipolar cells (Shiells and Falk, 1992; Hashimoto et al., 1997).

Activation of mGluR may either enhance or depress neurotransmitter release (see, e.g., Herrero et al., 1996), reflecting the diversity of mechanisms involved. mGluR may modulate neurotransmitter release resulting from inhibition of presynaptic Ca^{2+} channels (Herrero et al., 1996), modulation of presynaptic K^+ channels (Herrero et al., 1992; Saugstad et al., 1996), or direct effects on proteins of the exocytotic machinery (Scanziani et al., 1995).

Retinal amacrine cells are a structurally and functionally diverse group of cells that modulate the flow of visual information from the photoreceptors towards the ganglion cells that form the optic nerve. These cells express ionotropic and metabotropic receptors for glutamate released from bipolar cells (Duarte et al., 1998). A

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subpopulation of amacrine cells in the inner nuclear layer (INL) of the rat retina was found to express mRNAs for mGluR1, mGluR2, mGluR4, and mGluR7 (Hartveit et al., 1995). Immunocytochemistry studies revealed that mGluR2 is localized mainly in the processes of rat amacrine cells, postsynaptic to cone and rod bipolar cells, and staining with an antibody against mGluR2/3 gave a similar distribution in cat amacrine cells. The localization of mGluR4 immunoreactivity was similar to that of mGluR2 but was more widely distributed through the inner plexiform layer (Famiglietti, 1983; Koulen et al., 1996; Cai and Pourcho, 1999). mGluR7 receptors were also localized in neurites of amacrine cells, postsynaptic to ''OFF'' and ''ON'' cone bipolar cells synapses (Brandstätter et al., 1996), and an antibody against mGluR1α was found to label the processes of rat amacrine cells with cell bodies located in the middle of the INL (Peng et al., 1995). In the rat retina the processes containing mGluR1α and mGluR5a immunoreactivity are postsynaptic to OFF-type and ON-type cone bipolar cells and to rod bipolar cells (Koulen et al., 1997), whereas in the cat retina there is no mGluR1α immunoreactivity in elements postsynaptic to rod bipolar cells (Cai and Pourcho, 1999).

Although retinal amacrine cells express metabotropic glutamate receptors, their physiological role remains largely unknown (Duarte et al., 1998). Because mGluR are known to modulate neurotransmitter release (see above), we investigated their effect on the release of [^{3}H]acetylcholine ([^{3}H]ACh) from cultured amacrine-like neurons. Our results indicate that these cells are endowed with group III mGluR, which inhibit L- and N-type voltage-gated Ca^{2+} channels (VGCC) coupled to the release of [^{3}H]ACh. This inhibitory pathway was suppressed upon activation of group I or group II mGluR.

Abbreviations

ACh	acetylcholine
L-AP4	L(+)-2-amino-4-phosphonobutyric acid
BME	basal medium of Eagle
cAMP	cyclic AMP
ω-CgTx GVIA	ω-Conotoxin GVIA
Ch	choline
DCG-IV	(2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine
DHPG	(S)-3,5-dihydroxyphenylglycine
INL	inner nucleus layer
mGluR	metabotropic glutamate receptors
MSOP	(RS)-α-methylserine-O-phosphate
PTX	pertussis toxin
RO 20-1724,	4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone
TPB	tetrapheylboron
VGCC	voltage gated calcium channels

MATERIALS AND METHODS

Materials

Fetal calf serum was purchased from Biochrom KG (Berlim, Germany) and trypsin from GIBCO (Paisley, U.K.). ω-Conotoxin GVIA (ω-CgTx GVIA) was obtained from Peninsula Laboratories Europe (Marseyside, U.K.), and nitrendipine was a kind gift of Dr. G. Terstappen (Bayer A.G., Germany). [methyl-^{3}H]Choline chloride (85.0 Ci/mmol) was obtained from Amersham International (Amersham, U.K.). L(+)-2-amino-4-phosphonobutyric acid (L-AP4), (2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV), 3,5-dihydroxyphenylglycine (DHPG), and (RS)-α-methylserine-O-phosphate (MSOP) were obtained from Tocris Cookson Inc (Bristol, U.K.). The 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20–1724) and KT-5720 were purchased from Biomol (Plymouth Meeting, PA), and forskolin was from RBI (Natick, MA). All other reagents were from Sigma Chemical Co. (St. Louis, MO) or from Merck (Darmstadt, Germany).

Preparation and Culture of Chick Retina Cells

Primary cultures of chick retinal neurons from 8-day-old chick (white leghorn) embryos were obtained as described previously (Duarte et al., 1992). Briefly, the retinas were dissected free from other ocular tissues and incubated for 15 min at 37°C, in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution supplemented with 0.1% trypsin. The digested tissue was centrifuged at 140g_{av}, for 1 min, and the pellet was mechanically dissociated in basal medium of Eagle (BME), buffered with 20 mM HEPES and 10 mM $NaHCO_3$ and supplemented with 5% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). After the appropriate dilution, the cells were plated at a low density (0.4×10^6 cells/cm^2), in 35 mm plastic petri dishes coated with poly-D-lysine (0.1 mg/ml), for [^{3}H]ACh release experiments, or at high density (2×10^6 cells/cm^2), on six-well cluster plates also coated with poly-D-lysine (0.1 mg/ml), for cyclic AMP (cAMP) measurements. The cells were kept in culture for 5 days, at 37°C, in a humidified atmosphere of 95% air/5% CO_2 before the experiments. An antibody against choline acetyltransferase stained 81.2% ± 2.82% of the cells present in a preparation similar to the one used in the present experiments (Carvalho et al., 1998; Santos et al., 1998a), indicating that cultures of chick retinal neurons are enriched in cholinergic amacrine-like cells (Voigt, 1986; Wässle and Boycott, 1991).

ACh Release

Incubation with [^{3}H]choline. Low-density cultures of chick retinal neurons were incubated in Na^+-medium (in mM: 132 NaCl, 4 KCl, 1.4 $MgCl_2$, 1.2 H_3PO_4, 1

$CaCl_2$, 6 glucose, 10 HEPES-Na, pH 7.4) supplemented with 25 nM methyl-[^{3}H]choline ([^{3}H]Ch; 2.5 μCi/ml) for 45 min at 37°C. Incubation with [^{3}H]Ch and the release experiments were carried out in the presence of 50 μM eserine sulphate to prevent acetylcholine degradation. After loading, the cells were washed three times with Na^+ medium without radiolabelled compound, and further incubated for 15 min at 37°C in the same medium.

Release of [^{3}H]ACh and [^{3}H]Ch. The release of [^{3}H]ACh was measured using a superfusion method previously described (Drejer et al., 1987; Duarte et al., 1992). Briefly, the 35 mm petri dishes were covered with a piece of a nylon mesh (100 μm) and placed in a holder, where the dishes were maintained slightly inclined (5°). By using two peristaltic pumps, prewarmed (37°C) media with the desired composition were delivered to the top of the dishes (2 ml/min), and the superfusate was collected at the lower part of the petri dishes, every 1 min, with a Gilson FC-204 fraction collector. The cells were perfused for 10 min with Na^+ medium containing 20 mM ChCl (NaCl was isoosmotically replaced by 20 mM ChCl) and were further washed for 15 min with Na^+ medium, supplemented with 10 μM hemicholinium-3, to prevent Ch uptake. After this postloading period, the perfusion medium was changed to media with desired composition, and the samples were collected every 1 min. At the end of each experiment the cells were perfused with 0.2 M HCl for 5 min to collect the remaining intracellular neurotransmitter. From each sample, a 300 μl aliquot was collected to determine the [^{3}H]ACh present, and the remaining radioactivity was measured using Universol scintillation cocktail (ICN, Irvine, CA) and a Packard 2000 Spectometer provided with disintegrations per minute correction.

Separation of [^{3}H]ACh from [^{3}H]Ch. The separation of [^{3}H]ACh from [^{3}H]Ch, from the total tritium outflow was carried out as described by Rand and Johnson (1981). In brief, a 300 μl aliquot of each collected sample from the superfusate was incubated with 60 μl of assay mixture for phosphorylation of choline [80 mM $MgCl_2$, 12.5 mM ATP, 300 mM glycine buffer pH 8.0, 0.062 U/ml choline kinase (EC 2.7.32)] for 30 min at 37°C. After incubation, the samples were transferred into scintillation vials containing a 1:9 (0.5 ml:4.5 ml) mixture of tetraphenylboron (TPB) solution (30 g/liter in isoamyl alcohol) and toluene cocktail (5 g PPO and 0.2 g POPOP/liter). The vials were roughly shaken, allowing the extraction of [^{3}H]ACh into the organic phase of the scintillation cocktail by TPB, and the radioactivity was counted. The phosphorylated [^{3}H]Ch remains in the water phase and is not counted.

cAMP accumulation. High-density cultures of chick retinal neurons were washed three times with Na^+ medium and preincubated, in the same medium, with 25 μM RO-20–1724, a selective inhibitor of cAMP-specific phosphodiesterase (Nicholson et al., 1991) for 15 min at 37°C. The retinal cells were stimulated with 10 μM forskolin for 5 min or with 25 KCl for 1 min in the presence of adenosine deaminase (2 U/ml). The intracellular accumulation of cAMP under resting conditions was determined by incubating the cells in Na^+ medium, for 1 or 5 min. The reaction was stopped by removing the stimulation media, followed by addition of 1 ml ice-cold lysis buffer (50 mM Tris-HCl, 4 mM EDTA, pH 7.5). The cells were then scrapped and incubated for 4 min at 90°C, and the cAMP content of the samples was determined using a commercial cAMP radioimmunoassay kit (Amersham International, Buckinghamshire, U.K.).

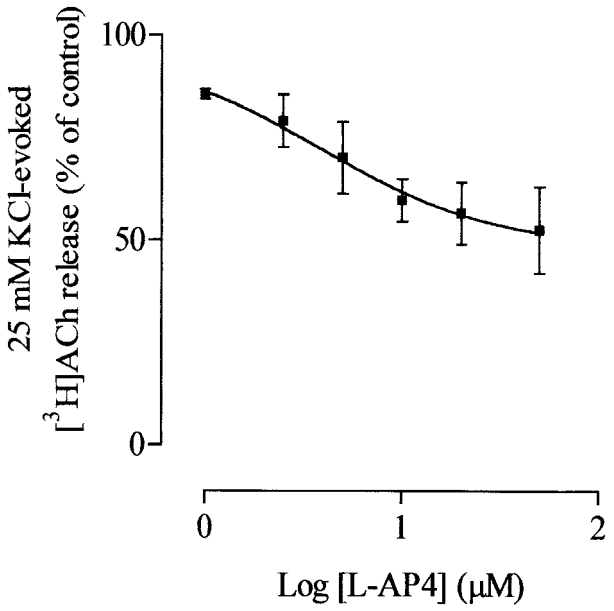


Fig. 1. Dose-dependent effect of the metabotropic glutamate receptor agonist L-AP4 on KCl-evoked [^{3}H]ACh release. The cells were preincubated with the indicated concentration of L-AP4 before depolarization for 1 min with 25 mM KCl, in 1 mM $CaCl_2$-containing medium supplemented with 50 μM eserine and the tested L-AP4 concentration. The results are expressed as a percentage of the control release, evoked by 25 mM KCl in the absence of L-AP4, and are means ± SEM for three or four experiments carried out in independent preparations.

Other Methods

Results are presented as means ± SEM of the number of experiments indicated. Statistical significance was determined using one-way ANOVA, and the differences between treatments were evaluated using the Bonferroni test.

RESULTS

Modulation of [^{3}H]ACh Release by Metabotropic Glutamate Receptors

Depolarization of cultured chick retina cells with KCl stimulates a Ca^{2+}-dependent release of [^{3}H]ACh,

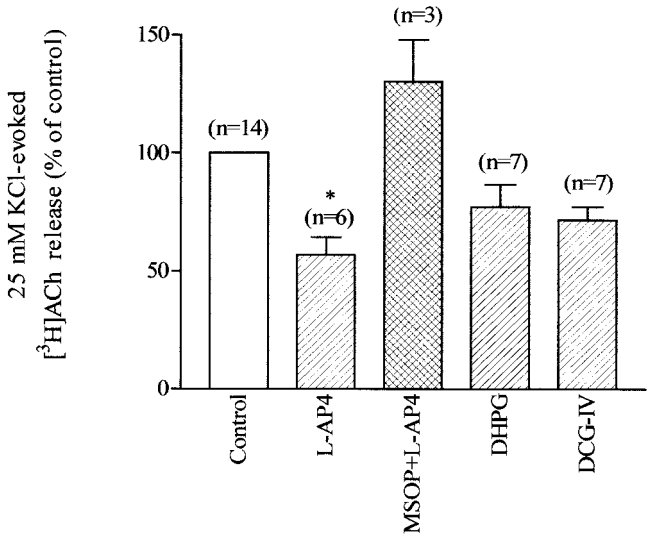


Fig. 2. Effect of the metabotropic glutamate receptor agonists L-AP4, DCG-IV, and DHPG on the release of [^{3}H]ACh induced by KCl. The cells were stimulated for 1 min with 25 mM KCl in 1 mM Ca^{2+}-containing Na^+ medium, supplemented with 50 μM eserine. The cells were incubated with 20 μM L-AP4, in the presence or in the absence of 500 μM MSOP, with 1 μM DCG-IV or with 50 μM DHPG, for 2 min, before KCl depolarization. The results are expressed as a percentage of the control release, evoked by 25 mM KCl in the absence of any agonists and are means ± SEM for the indicated number of experiments, performed in independent preparations. *$P < 0.05$, Bonferroni's multiple comparison test.

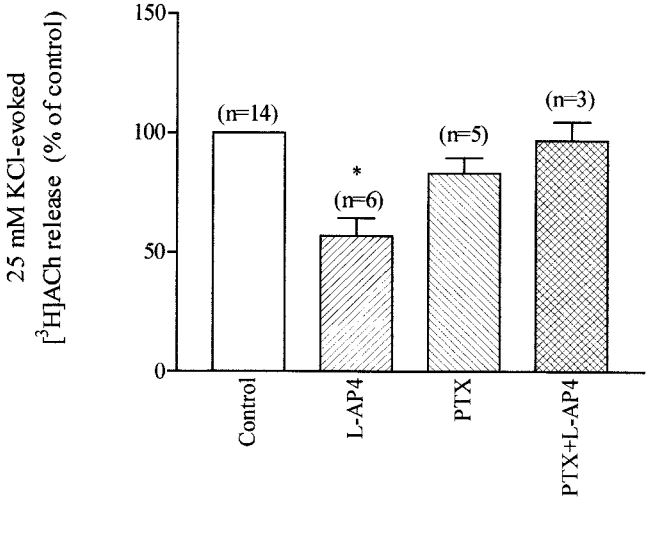


Fig. 3. Effect of pertussis toxin (PTX) on the modulation of [^{3}H]ACh release by L-AP4. The cells were incubated for 17 hr with PTX (50 ng/ml) in culture medium, and the release experiments were performed without the toxin. When the effect of L-AP4 was tested, the cells were incubated with the agonist at 20 μM for 2 min before depolarization with 25 mM KCl. The results are expressed as a percentage of the control [^{3}H]ACh release and are means ± SEM for the indicated number of experiments, performed in independent prepartions. *$P < 0.05$, Bonferroni's multiple comparison test.

with an EC_{50} of about 22 mM (Santos et al., 1998a). Perfusion of the cells with 25 mM KCl for 1 min evoked the release of 2.3 ± 1.1% of the total intracellular [^{3}H]ACh, in Na^+ medium containing 1 mM $CaCl_2$. The agonist of group III mGluR L-AP4 (Conn and Pin, 1997) reduced 25 mM KCl-evoked [^{3}H]ACh release, in a dose-dependent manner, with an IC_{50} of 3.9 μM (Fig. 1). After a 2 min preincubation with 20 μM L-AP4, the release of [^{3}H]ACh was maximally reduced to 56.7% ± 7.5% of the control (Fig. 2). MSOP (500 μM), an antagonist of group III mGluR (Thomas et al., 1996), reversed the inhibitory effect of L-AP4 on [^{3}H]ACh release ($P < 0.001$).

When the retina cells in culture were preincubated with 50 ng/ml pertussis toxin (PTX) for 17 hours, the inhibitory effect of L-AP4 on [^{3}H]ACh release evoked by 25 mM KCl was no longer observed (Fig. 3). The [^{3}H]ACh release observed under these experimental conditions (96.5% ± 7.8% of the control) was not significantly different from the control release evoked by 25 mM KCl in the cells preincubated or not with PTX ($P > 0.05$). These results indicate that group III mGluR modulates the release of [^{3}H]ACh by activating PTX-sensitive G proteins. In contrast with the effect of L-AP4, stimulation of group I or group II mGluR, with 50 μM DHPG (Ito et al., 1992; Schoepp et al., 1994) and 1 μM DCG-IV (Hayashi et al., 1993; Ishida et al., 1993), respectively, had a small effect on [^{3}H]ACh release evoked by 25 mM KCl, which did not reach statistical significance ($P > 0.05$) (Fig. 2).

Group III mGluR Selectively Modulate the Activity of Ca^{2+} Channels

The release of [^{3}H]ACh from cultured chick amacrine-like neurons is due to Ca^{2+} entry through L- and N-type voltage-gated Ca^{2+} channels (VGCC) and through as yet uncharacterised Ca^{2+} channels (Santos et al., 1998a). To evaluate whether VGCC inhibition contributes to the observed effect of group III mGluR on [^{3}H]ACh release, we investigated the effect of blockers of L- and N-type VGCC on [^{3}H]ACh release in the absence and in the presence of L-AP4. In the presence of 300 nM nitrendipine, a concentration that produces maximal inhibition of L-type VGCC (McCleskey et al., 1986; Santos et al., 1998a), the release of [^{3}H]ACh induced by 25 mM KCl was reduced to 78.3% ± 6.5% of control (Fig. 4A). No significant difference ($P > 0.05$) was found between [^{3}H]ACh release evoked by KCl in cells stimulated with L-AP4 or with L-AP4 and nitrendipine. These results indicate that activation of L-AP4-sensitive receptors inhibits [^{3}H]ACh release evoked by Ca^{2+} entry through L-type VGCC. However, L-AP4 still inhibited the release

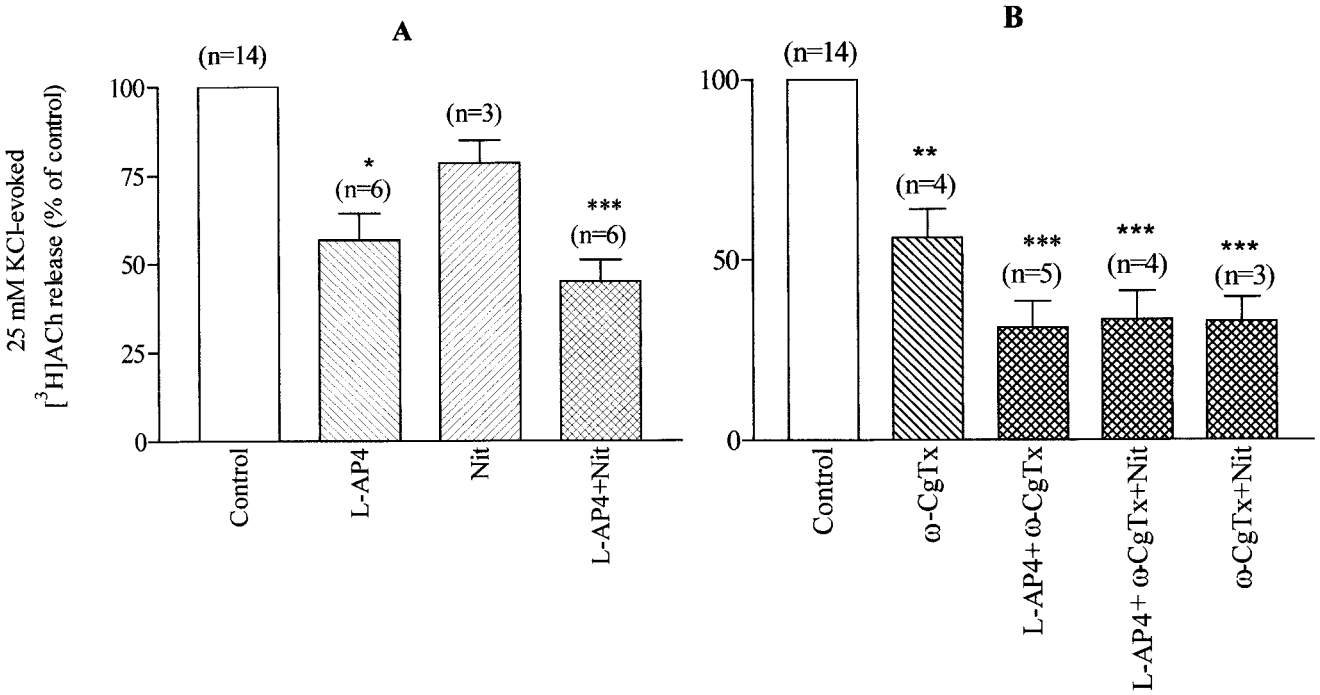


Fig. 4. Effect of L-AP4 on nitrendipine-sensitive (**A**) and on ω-CgTx GVIA-sensitive (**B**) VGCC coupled to [^{3}H]ACh release. The cells were perfused for 2 min in the absence or in the presence of L-AP4 (20 μM) and/or nitrendipine (300 nM), as indicated, and were then depolarized with 25 mM KCl for 1 min in the presence of the same drugs. When the effect of the ω-Cgtx GVIA was tested, the cells were preincubated with 30 nM of the toxin for 1 hr, during the period of radiollabeling, but the toxin was not present during superfusion. The experiments were conducted in a medium containing 1 mM $CaCl_2$ and supplemented with 50 μM eserine. The results are expressed as a percentage of control [^{3}H]ACh release evoked by 25 mM KCl, and are means ± SEM for the indicated number of experiments, performed in independent preparations. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Bonferroni's multiple comparison test.

of [^{3}H]ACh evoked by KCl in the presence of nitrendipine ($P < 0.05$), indicating that the group III mGluR can affect the release by a second mechanism, not involving L-type VGCC.

A saturating concentration (30 nM; Santos et al., 1998a) of the N-type VGCC blocker ω-CgTx GVIA (Olivera et al., 1985) inhibited [^{3}H]ACh release evoked by 25 mM KCl, to 56.0% ± 8.0% of the control, in agreement with previous results showing that these channels play a major role in the regulation of [^{3}H]ACh release by cultured retina cells (Santos et al., 1998a). When the cells were preincubated with ω-CgTx GVIA together with L-AP4, the release of [^{3}H]ACh (31.1% ± 7.3% of the control) was significantly lower ($P < 0.05$) than that measured in the presence of the mGluR agonist alone (56.7% ± 7.5% of control; Fig. 4). The effects of L-AP4 and ω-CgTx GVIA on KCl-evoked [^{3}H]ACh release are far from being additive, suggesting that the N-type VGCC are partly affected by activation of group III mGluR. The N-type VGCC are likely to represent the nitrendipine-insensitive component of the effect of L-AP4 on [^{3}H]ACh release; indeed, the mGluR agonist did not affect neurotransmitter release after preincubation of the cells with nitrendipine and ω-CgTx GVIA (Fig. 4B).

When saturating concentrations of nitrendipine (300 nM) and ω-CgTx GVIA (30 nM; Santos et al., 1998a) were tested together, the release of [^{3}H]ACh evoked by 25 mM KCl was inhibited to 33.0% ± 6.4% of control (Fig. 4B). This inhibition was not enhanced by preincubation of the cells with L-AP4 (33.3% ± 7.9% of control), suggesting that stimulation of the cells with L-AP4 does not affect the activity of non-L and non-N VGCC coupled to the release of [^{3}H]ACh.

Effect of L-AP4 on [^{3}H]ACh Release Is Not Due to Inhibition of Adenylate Cyclase

It is known that L-AP4-sensitive mGluR are negatively coupled to cAMP formation (Conn and Pin, 1997). To determine whether the effect of L-AP4 on [^{3}H]ACh release could be attributed to changes in cAMP levels and to a consequent decrease in protein kinase A activity, we determined the effect of the mGluR agonist on cAMP production evoked by forskolin. Furthermore, we measured the effect of protein kinase A inhibition on [^{3}H]ACh release evoked by KCl.

Basal cAMP levels were not significantly ($P > 0.05$) changed either by 20 μM L-AP4 or by depolaring the

cells with 25 mM KCl for 1 min (Fig. 5A). When the cells were depolarized by 25 mM KCl in the presence of 20 μM L-AP4, after preincubation with the agonist for 1 min, no effect on the amount of cAMP present in this cells was observed compared to the basal value. In contrast, 1 μM DCG-IV decreased the basal cAMP levels, from 0.62 ± 0.04 to 0.32 ± 0.04 pmol/10^6 cells (Fig. 5A).

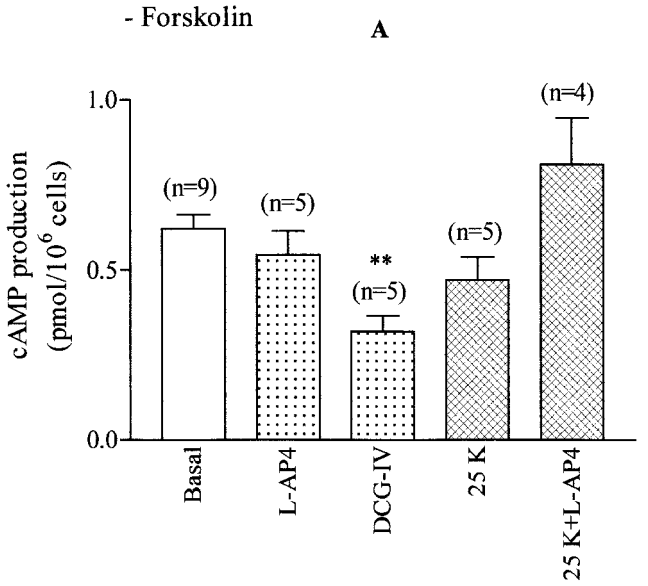
Stimulation of cultured retina cells with 10 μM forskolin for 5 min increased the intracellular accumulation of cAMP, from a value of 6.62 ± 0.12 pmol/10^6 cells to 34.94 ± 2.78 pmol/10^6 cells (Fig. 5B). Activation of group III mGluR with 20 μM L-AP4, did not affect significantly the accumulation of cAMP induced by forskolin (34.00 ± 4.42 pmol/10^6 cells). In contrast, 1 μM DCG-IV partly inhibited the effect of forskolin on the production of cAMP, down to 15.11 ± 3.60 pmol/10^6 cells, and this effect was reversed to some extent after preincubation of the cells with PTX (23.40 ± 2.01 pmol/10^6 cells). The cAMP accumulation under the latter experimental conditions was not significantly different ($P > 0.05$) from that measured in cells stimulated with forskolin after incubation with PTX.

Although the results in Figure 5 indicate that the effect of L-AP4 on [^{3}H]ACh cannot be attributed to an inhibition of adenylate cyclase, we also investigated whether protein kinase A (PKA) inhibition affects the release of the neurotransmitter. The PKA inhibitor KT-5720 (500 nM; Kase et al., 1987) was without effect on [^{3}H]ACh release evoked by 25 mM KCl (Fig. 6).

Cross-Talk Between mGluR

Although agonists of group I and II mGluR did not have a significant direct effect on the release of [^{3}H]ACh, the results in Figure 5 and our previous results (Duarte et al., 1996) suggest that they are present in the cultured retinal neurons. Because these receptors are also expected to be activated by glutamate when the neurotransmitter activates group III mGluR, we investigated whether group I and group II receptors could modulate the effect of L-AP4-sensitive receptors on [^{3}H]ACh release.

The inhibitory effect of 20 μM L-AP4 on [^{3}H]ACh release evoked by 25 mM KCl was abolished when the



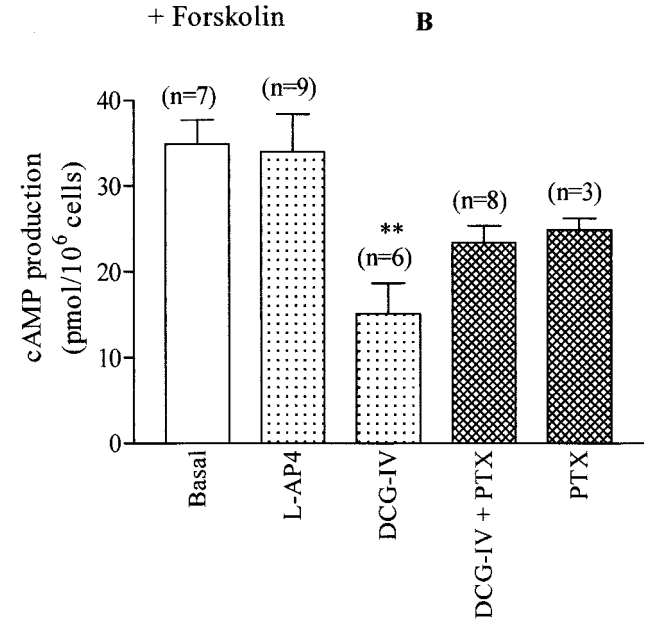


Fig. 5. Effect of mGluR agonists on cAMP levels in the presence or in the absence of forskolin. Cultured chick retina cells were washed with Na^+ medium and preincubated in the same medium supplemented with 25 μM RO20–174 for 15 min at 37°C. **A:** The cells were then mantained in the Na^+ medium, with or without 20 μM L-AP4 or 1 μM DCG-IV, for 5 min, as indicated. Alternatively, the cells were depolarized with 25 mM KCl for 1 min in a medium containing 1 mM $CaCl_2$, in the presence or in the absence of 20 μM L-AP4. In these experiments the cells were preincubated with L-AP4 for 1 min, before KCl depolarization. Control experiments revealed that the basal accumulation of cAMP was the same in cells incubated in Na^+ medium for 1 or 5 min (not shown). **B:** The cells were incubated with 10 μM forskolin for 5 min in the presence or in the absence of the indicated mGluR agonists at the indicated concentrations. Pretreatment with PTX (50 ng/ml) was carried out by incubating the cells with the toxin (50 ng/ml) for 17 hr in cultured medium. All the experiments were carried out in the presence of ADA (2 U/ml). The results are means ± SEM for the indicated number of experiments, carried out in independent prepartions $^{**}P < 0.01$, Bonferroni's multiple comparison test.

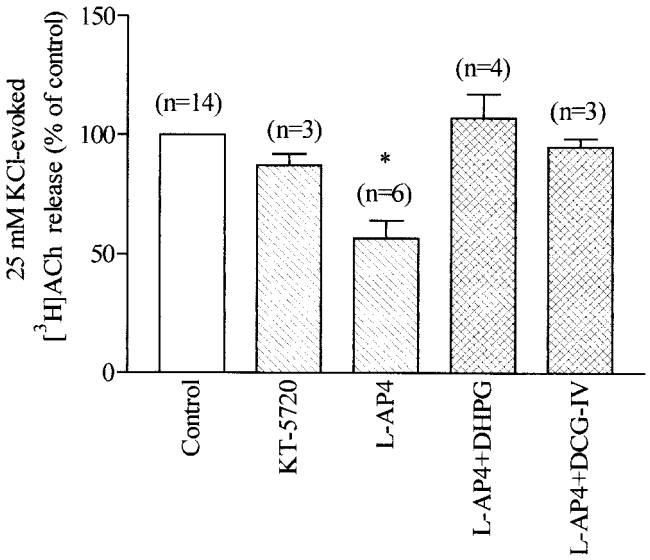


Fig. 6. Role of PKA and interaction between mGluR in the modulation of [^{3}H]ACh release. The cells were preincubated or not with the indicated agonists for 2 min, before depolarization with 25 mM KCl under the same experimental conditions. The drugs were used at the following concentrations: 500 nM KT-5720, 20 μM L-AP4, 50 μM DHPG and 1 μM DCG-IV. The experiments were carried out in 1 mM Ca^{2+}-containing Na^+ medium, suplemented with 50 μM eserine. The results are expressed as a percentage of control, obtained after KCl depolarization, and are means ± SEM for the indicated number of experiments, performed in independent preparations. *$P < 0.05$, Bonferroni's multiple comparison test.

cells were stimulated with L-AP4 together with 50 μM DHPG (Fig. 6). Moreover, costimulation of group II and group III mGluR, with 1 μM DCG-IV and 20 μM L-AP4, respectively, also prevented the inhibitory effect of L-AP4 on the release of [^{3}H]ACh evoked by 25 mM KCl (Fig. 6). Although DCG-IV was recently shown to act as an antagonist of group III mGluR, the IC_{50}s determined are higher (above 20 μM; Barbet et al., 1998) than the concentration used in the present work (1 μM). Therefore, the effect of DCG-IV in preventing the inhibition of [^{3}H]ACh release by L-AP4 is unlikely to be due to an action as antagonist of group III mGluR. Our results indicate that there is cross-talk between mGluR in retina cells, which modulates the ability of group III mGluR to affect [^{3}H]ACh release.

DISCUSSION

Our results show that L-AP4-sensitive group III mGluR inhibit the KCl-evoked release of [^{3}H]ACh from cultured amacrine-like neurons. The IC_{50} for L-AP4-induced inhibition of [^{3}H]ACh release was 3.9 μM, which is similar to the reported EC_{50} for activation of mGluR4a, mGluR6, and mGluR8 by the same agonist (reviewed by Conn and Pin, 1997). The inhibition of [^{3}H]ACh release by L-AP4 reported here may arise from the blockade of L- and N-type VGCC, through the activation of a PTX-sensitive G protein. Although group III mGluR are generally coupled to the inhibition of adenylyl cyclase (Conn and Pin, 1997), this is not responsible for the effect of L-AP4 on [^{3}H]ACh release reported here for two reasons: 1) L-AP4 was without effect on cAMP production stimulated by forskolin and 2) PKA inhibition with KT-5720 was without effect on KCl-evoked [^{3}H]ACh release.

To our knowledge this is the first report concerning the presynaptic effects of mGluR on depolarization evoked [^{3}H]ACh release. The mGluR are also known to serve as presynaptic autoreceptors at glutamatergic synapses and as presynaptic heteroreceptors at GABAergic and dopaminergic synapses (Conn and Pin, 1997; Verma and Moghaddam, 1998). However, the mechanism(s) by which mGluR modulate neurotransmitter release depends on the receptors involved and on the region of the nervous system. Group III mGluR may regulate neurotransmitter release by decreasing calcium entry though VGCC (Stefani et al., 1996).

Cultured chick amacrine-like neurons are endowed with L-, N-, and P/Q-type VGCC (Wei et al., 1989; Duarte et al., 1992, 1996). We have shown that the release of [^{3}H]ACh evoked KCl in this preparation is inhibited by nitrendipine and ω-CgTx GVIA, indicating that it is dependent of Ca^{2+} entry through L- and N-type VGCC. Activation of group III mGluR with L-AP4 inhibited mainly the component of [^{3}H]ACh release that was dependent on Ca^{2+} influx through L-type VGCC; the L-AP4-resistant [^{3}H]ACh release was not affected by nitrendipine. However, part of the effect of L-AP4 could not be attributed to the inhibition of L-type VGCC coupled to [^{3}H]ACh release, and this component is likely to be due to a partial inhibition of ω-CgTx GVIA-sensitive VGCC. The release of [^{3}H]ACh from amacrine-like neurons is also partly dependent on Ca^{2+} entry through as yet unidentified channels, insensitive to nitrendipine, ω-CgTx and to ω-agatoxin IVA (Santos et al., 1998a). However, our results indicate that L-AP4 does not affect the component of [^{3}H]ACh release resulting from Ca^{2+} entry through these channels. Therefore, we can conclude that, in cultured chick amacrine-like neurons, the activation of the group III mGluR inhibits [^{3}H]ACh release coupled to the influx of Ca^{2+}through L- and N-type VGCC, suggesting that these channels are selectively inhibited by group III mGluR. Group II mGluR were also shown to inhibit directly the activity of L-type VGCC by a mechanism involving the activity of G_i or G_o proteins in cultured cerebellar granule cells (Chavis et al., 1994).

The present results indicating that group III mGluR modulate the release of [^{3}H]ACh in a PTX-sensitive manner, by inhibiting L- and N-type Ca^{2+} channels, contrast with the events responsible for the inhibition of [^{3}H]ACh by adenosine A_1 receptors in cultured chick amacrine-like neurons reported earlier (Santos et al., 1998b). In this preparation the release of [^{3}H]ACh is also under tonic inhibition by adenosine A_1 receptors that act through activation of PTX-sensitive G proteins. However, in contrast to the results reported here, the effect of A_1 receptors on [^{3}H]ACh release is exclusively due to a selective inhibition of N-type VGCC (Santos et al., 1998b). Although it is not clear at the moment why the inhibitory adenosine A_1 receptors selectively inhibit N-type VGCC, in contrat to group III mGluR, which target L- and N-type VGCC coupled to the release of [^{3}H]ACh, this difference may be due to a differential distribution of the receptors within the synapses, or they may be localized in different synapses.

The effect of L-AP4 on [^{3}H]ACh release was fully antagonized by PTX, a toxin that irreversibly inhibits G_i or G_o proteins by ADP-ribosylation of the α subunit, preventing the coupling of the mGluR with the effector system (Dolphin and Prestwich, 1985). Therefore, it is possible that the $\alpha_{i/o}$ and/or $\beta\gamma$ subunits of the activated G protein directly inhibit the activity of L- and N-type VGCC (see, e.g., Toselli et al., 1989; Haws et al., 1993; Ikeda et al., 1995; Ikeda, 1996; Herlitze et al., 1996; De Waard et al., 1997; Dolphin, 1998).

The activation of group III mGluR has been shown to inhibit adenylate cyclase in brain slices and neuronal cultures, in studies in which the enzyme was usually stimulated with forskolin (for references see Conn and Pin, 1997). This inhibition and the subsequent decrease in cAMP levels reduce the activation of PKA and the phosphorylation of its substrates. The α_1 subunits of L-and N-type VGCC are targets of PKA (Hell et al., 1994, 1995), and the activity of these channels may increase upon phosphorylation (Gao et al., 1997; Kavalali et al., 1997; see also Hell et al., 1995, for other references). Therefore, a decrease in the phosphorylation of L-type VGCC, following inhibition of group III mGluR, could account for the observed inhibition of [^{3}H]ACh release. However, this is certainly not the mechanism involved because activation of group III mGluR did not affect cAMP levels in depolarized retina cells, and also the PKA inhibitor KT-5720 was without effect on KCl-evoked [^{3}H]ACh release. The lack of effect of L-AP4 on cAMP formation in cultured retina cells is similar to the lack of effect, or to the small effect, of the group III mGluR agonist on adenylate cyclase activity in several cell types expressing mGluR4 mRNA (Prezeau et al., 1994). In the chick retina the effect of group III mGluR on adenylate cyclase is developmentally regulated, being maximal during the period of formation of the plexiform layers and the synapses (E12–E17) and decreasing afterwards (Sampaio and Paes-de-Carvalho, 1998). The lack of effect of L-AP4 on forskolin-stimulated cAMP accumulation observed here indicates that the cells present in the cultures express group III mGluR functionally similar to those present in the mature retina.

Besides expressing group III mGluR, the cultured chick amacrine-like neurons also express functional DHPG (group I)- and DCG-IV (group II)-sensitive receptors (see also Duarte et al., 1996; Sampaio and Paes-de-Carvalho, 1998). Immunocytochemistry experiments showed that indeed rat cholinergic amacrine cells express mGluR2, a group II mGluR (Koulen et al., 1996). Although the latter receptors did not affect significantly the release of [^{3}H]ACh release evoked by KCl, they reversed the inhibitory effect of group III mGluR. These results indicate that cholinergic amacrine-like neurons may coexpress more than one type of mGluR, which may interact functionally with each other within the terminal.

The signalling mechanisms involved in the effect of DHPG and of DCG-IV on group III mGluR function are likely to be different. Indeed, group I mGluR are coupled to the activation of PLC in cultured retinal neurons (Duarte et al., 1996), thereby generating diacylglycerol, active on protein kinase C (PKC), and inositol 1,4,5-trisphosphate. PKC activation is likely to be involved in the suppression of the L-AP4 inhibitory action, possibly by inhibition of coupling of mGluR to G proteins, as previously reported (Herrero et al., 1996; Macek et al., 1998). The PKC-mediated suppression of presynaptic inhibitory actions has also been observed for adenosine (A_1) receptors (Thompson et al., 1992), α_1-adrenergic receptors (Scanziani et al., 1993), mGluR (Swartz et al., 1993), and $GABA_B$ receptors (Dutar and Nicoll, 1988). Activation of group II mGluR decreases the resting cAMP accumulation in retina cells and cAMP production in response to adenylate cyclase stimulation with forskolin (Fig. 4). Although the effect of DCG-IV on adenylate cyclase activity was mediated through activation of a PTX-sensitive G protein, it is not clear whether it is the consequent reduction in PKA activity that suppresses the inhibitory effect of L-AP4 on [^{3}H]ACh release.

In conclusion, our results show that in cultured amacrine-like neurons, KCl-evoked [^{3}H]ACh release is directly modulated by group III mGluR, possibly through a direct inhibitory effect on the activity of L- and N-type type VGCC. Cholinergic amacrine cells make feed-forward synapses to ganglion cells (Famiglietti, 1983). The activation of group III mGluR by glutamate released by bipolar cells decreases the release of [^{3}H]ACh and, therefore, may modulate the firing rate of the postsynaptic ganglion cells. A subpopulation of the cholinergic amacrine cells also contain GABA, although the localisation

of the two transmitters within the cells is not clearly established (Santos et al., 1998a). It will be of interest to determine whether the release of GABA from the cholinergic amacrine neurons is also modulated by mGluR.

The cholinergic ''starburst'' amacrine cells and GABAergic amacrine cells may also play an important role on retinal directional selectivity, a process whereby directionally selective retinal ganglion cells produce robust responses for motion in certain directions, but not in others (Barlow and Levick, 1965; Duarte et al., 1999). Insofar as mGluR modulate ACh release, it will be of interest to determine whether they contribute to directional selectivity.

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