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$[Ca^{2+}]_i$ Regulation by Glutamate Receptor Agonists in Cultured Chick Retina Cells

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The effect of glutamate receptor agonists on the intracellular free calcium concentration ($[Ca^{2+}]_i$), measured with Indo-1, was studied in populations of cultured chick embryonic retina cells. The agonists of the ionotropic glutamate receptors, *N*-methyl-D-aspartate (NMDA), kainate, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) increased the $[Ca^{2+}]_i$ through a composite effect, comprising Ca^{2+} permeating the receptor-associated channels, and Ca^{2+} entering through voltage-gated Ca^{2+} channels. Furthermore, the $[Ca^{2+}]_i$ responses to NMDA and AMPA also involved Ca^{2+} release from intracellular stores, which could not be mobilized by stimulation of the metabotropic receptor.

Glutamate receptors $[Ca^{2+}]_i$ Ca^{2+} channels ACPD receptors Cultured retina cells

INTRODUCTION

In the retina, as in the cerebral cortex, glutamate is the major excitatory neurotransmitter in both plexiform layers (Barnstable, 1993). Glutamate activates ionotropic receptors and also receptors coupled to guanine nucleotide-binding proteins (reviews by Seeburg, 1993; Schoepp & Conn, 1993; Hollmann & Heinemann, 1994). The ionotropic glutamate receptors have been divided into three families according to their pharmacological, molecular biological and electrophysiological properties: the NMDA, AMPA and kainate receptors. The NMDA receptor is largely permeable to Ca^{2+}, in addition to Na^+ and K^+ ions, and thus is expected to generate composite $[Ca^{2+}]_i$ signals due to Ca^{2+} influx through the receptor-associated channel and Ca^{2+} entering through voltage-sensitive Ca^{2+} channels (VSCCs) (Mayer & Miller, 1990). The kainate and AMPA receptors are generally regarded as low-Ca^{2+} permeability receptors (Mayer & Westbrook, 1987; Mayer & Miller, 1990). The activation of these receptors is thought to increase the $[Ca^{2+}]_i$ mainly via depolarization-induced opening of VSCCs. However, some Ca^{2+} permeable kainate and AMPA receptors have been described (e.g. Gilbertson *et al.*, 1991; Brorson *et al.*, 1992; Burnashev *et al.*, 1992a,b; Schneggenburger *et al.*, 1993). A subclass of the metabotropic glutamate receptors activates the phospholipase C, leading to the formation of inositol 1,4,5-trisphosphate which releases Ca^{2+} from intracellular stores (Courtney *et al.*, 1990; Irving *et al.*, 1990; Schoepp & Conn, 1993). The Ca^{2+} entry upon activation of the ionotropic glutamate receptors may also lead to the release of intracellular Ca^{2+} by CICR (Segal & Manor, 1992; Simpson *et al.*, 1993).

In the inner plexiform layer of the retina, the bipolar cells, which are glutamatergic (Barnstable, 1993), provide the excitatory input to the amacrine and ganglion cells. The amacrine cells have been found to be depolarized by excitatory amino acids (Slaughter & Miller, 1983), and glutamate and its agonists stimulate the release of γ-aminobutyric acid (GABA) and acetylcholine from amacrine cells (Hofmann & Möckel, 1991; Linn & Massey, 1991; Linn *et al.*, 1991; Duarte *et al.*, 1993; Alfonso *et al.*, 1982; Ferreira *et al.*, 1994). The amacrine cells have been shown to respond to both NMDA and non-NMDA receptor agonists (Linn & Massey, 1991; Linn *et al.*, 1991; Dixon & Copenhagen, 1992; Ferreira *et al.*, 1994).

Since the Ca^{2+} signals provided by activation of the glutamate receptors are diverse, they are likely to have distinct functions within the neurons. Thus, it is important to separate the $[Ca^{2+}]_i$ signals generated by each glutamate receptor agonist into its component parts. Therefore, we characterized in detail the different

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Abbreviations: 1*S*,3*R*-ACPD, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; L-AP3, L(+)-2-amino-3-phosphonopropionic acid; BME, Basal medium of Eagle (Earle's salts); $[Ca^{2+}]_i$, internal free calcium ion concentration; CICR, Ca^{2+}-induced Ca^{2+} release; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; 5,7-DCK, 5,7-dichlorokynurenic acid; DHP, 1,4-dihydropyridines; Indo-1/AM, acetoxymethyl ester of Indo-1; $InsP_1$, inositol monophosphate; $InsP_2$, inositol bisphosphate; $InsP_3$, inositol trisphosphate; $InsP_4$, inositol tetrakisphosphate; MK-801, (+)-5-methyl-10,11-dihydro-5 H-dibenzo[a,d] cyclohepten-5,10-imine maleate; NMDA, *N*-methyl-D-aspartate; NMG, *N*-methyl-D-glucamine; VSCCs, voltage-sensitive Ca^{2+} channels.

pathways of Ca^{2+} entry and the contribution of internal Ca^{2+} stores to the $[Ca^{2+}]_i$ signals triggered by activation of the glutamate receptors in cultures enriched in chick retinal amacrine-like neurons (Hofmann & Möckel, 1991).

MATERIALS AND METHODS

Materials

The Indo-1/AM was purchased from Molecular Probes Inc., Eugene, OR, U.S.A. Nitrendipine and thapsigargin were obtained from RBI, Natick, MA, U.S.A. and ω-Aga IVA from SMA, Herts, U.K. NMDA, kainic acid, AMPA, ACPD, CNQX, 5,7-DCK and L-AP3 were from Tocris Neuramin, Bristol, U.K. Nifedipine was a kind gift of Dr G. Terstappen, from Bayer A.G., Germany. Ionomycin was obtained from Calbiochem-Boehringer Corp., San Diego, CA U.S.A., and myo-[^{3}H]inositol from Amersham International, U.K. Antibiotics and fetal calf serum were obtained from Biological Industries, Beth Ha Emek, Israel, and trypsin from GIBCO, Paisley, U.K. MK-801 was a kind gift of Merck Sharp and Dohme, U.S.A. All other reagents were from Sigma, St Louis, MO, or Merck, Darmstadt, Germany.

Solutions

Stock solutions of Indo-1/AM, nitrendipine, nifedipine, CNQX, thapsigargin and ionomycin were made in dimethylsulfoxide. ω-Aga IVA, NMDA, kainic acid, AMPA, 1*S*,3*R*-ACPD, 5,7-DCK, L-AP3 and glutamate were kept in aqueous stocks.

The Na^+-salt solution used for $[Ca^{2+}]_i$ determinations contained (in mM): 132 NaCl, 4 KCl, 1 $CaCl_2$, 1.4 $MgCl_2$, 6 glucose and 10 HEPES-Na, pH 7.4. The NMG medium was prepared by iso-osmotic replacement of NaCl by NMG, but was otherwise identical. The Mg^{2+}-free Na^+ and NMG media were prepared by replacing isoosmotically $MgCl_2$ by the major salt. The accumulation of myo-[^{3}H]inositol phosphates was determined in Li^+ medium, identical to the Na^+-salt solution except containing 122 mM NaCl, 10 mM LiCl and 1.2 mM H_3PO_4. The osmolarity of all media was adjusted to 285 ± 5 mosM with the major salt.

Preparation and culture of chick retina cells

The retinas from 8-day-old chick (White Leghorn) embryos were dissected free from other ocular tissues, cut into small pieces, 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 (Mello, 1984). The digested tissue was centrifuged at 140 g_{av} for 1 min, and the pellet was resuspended in BME supplemented with 5% fetal calf serum. The tissue was then dissociated mechanically by 10–14 aspirations with a large-bore 5 ml glass pipette. The cells were cultured in 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). For Indo-1 fluorescence measurements the cells were seeded at a density of 0.6×10^6 cells/cm^2 on poly-L-lysine-coated glass coverslips. A preparation similar to this one has been proposed to be highly enriched in amacrine-like neurons (Huba & Hofmann, 1990), but all our preparations contained a small but significant percentage of neurons resembling bipolar cells. Measurements of the accumulation of inositol phosphates were carried out using cells plated at a density of 2×10^6 cells/cm^2, on 6-well cluster plates. Cells were cultured for 5 days at 37°C in a humidified atmosphere of 95% air/5% CO_2.

Primary cultures of chick retina flat cells (putative glial cells) were prepared as previously described (Adler *et al.*, 1982), with some modifications. In short, chick embryo retina cells were isolated by trypsin digestion, as described above, and resuspended in BME, buffered with 26 mM $NaHCO_3$, and supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were seeded on 6-well cluster plates at a density of 0.4×10^6 cells/cm^2, and cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2. After 7 days in culture, the network of neuronal clumps was removed using a fine stream of medium, leaving purified non-neuronal flat cells monolayers (Adler *et al.*, 1982).

Indo-1 loading and $[Ca^{2+}]_i$ measurements in populations of cells

Chick retina cells cultured on glass coverslips were incubated with 3 μM Indo-1/AM, in BME buffered with 20 mM HEPES. Dye loading was performed for 45 min at 37°C, and the cells were further incubated for 15 min in HEPES-buffered BME, in order to ensure a complete hydrolysis of the acetoxymehyl ester of Indo-1. The coverslips were rinsed with Na^+-salt solution, and thereafter mounted in a temperature-controlled cuvette as described (Duarte *et al.*, 1992, 1993). The Indo-1 fluorescence was measured using a computer-assisted Perkin Elmer LS-5B Luminescence Spectrometer, with excitation at 335 nm and emission at 410 nm, and using 5 nm slits. After 4 min pre-incubation, the cells were stimulated with the indicated glutamate receptor agonist. In establishing the proper concentration employed, we tested in all cases concentrations up two-fold higher than those used, and which already produced maximal effect. The $[Ca^{2+}]_i$ was calculated as described previously (Duarte *et al.*, 1992, 1993), by using 3 μM ionomycin to obtain the maximal fluorescence and 2 mM $MnCl_2$ to determine the autofluorescence.

Accumulation of myo-[^{3}H]inositol phosphates

The neuron-rich cultures and the cultured retinal flat cells were pre-incubated with myo-[^{3}H]inositol (5 μCi/ml) in inositol-free BME, buffered with 26 mM $NaHCO_3$, for 7 hr at 37°C. The cells were then washed three times with Na^+ buffer, containing (in mM): 132 NaCl, 4 KCl, 1.4 $MgCl_2$, 1 $CaCl_2$, 1.2 H_3PO_4, 6 glucose and 10 HEPES-Na, pH 7.4, and incubated in the same medium for 20 min at 37°C. Before stimulation, the cells

were further incubated for 10 min at 37°C in 10 mM Li^+ buffer (identical to the Na^+ buffer, except containing 122 mM NaCl and 10 mM LiCl). The cells were stimulated for 2 min with the indicated agonists and/or antagonists prepared in Li^+ buffer, and the reactions were stopped by removing the medium and adding ice-cold 10% trichloroacetic acid. The samples were kept frozen for later analysis.

Separation of myo-[^{3}H]inositol phosphates

The [^{3}H]inositol phosphates were extracted and separated as previously described (Berridge *et al.*, 1983), with some modifications. The cells were scraped from the wells and centrifuged at 15,800 *g* for 2 min at 4°C. The extract was removed, washed three times with water-saturated diethyl ether, and neutralized with 1 M NaOH before being applied to the anion-exchange columns. The pellets were solubilized in 1 M NaOH and analyzed for their protein content by the method of Bradford (1976), using bovine serum albumin as standard.

The water-soluble extracts were applied to polypropylene columns (BioRad) containing 0.8 ml of Dowex AG1-X8 resin (100–200 μm mesh, format form) previously equilibrated with distilled water. The columns were first washed with 12 ml of 5 mM myo-inositol followed by 12 ml of 60 mM ammonium formate/5 mM sodium tetraborate, to remove myo-[^{3}H]inositol and glycerophosphoinositides, respectively. The [^{3}H]inositol monophosphate ([^{3}H]$InsP_1$), [^{3}H]inositol bisphosphate ([^{3}H]$InsP_2$), [^{3}H]inositol trisphosphate ([^{3}H]$InsP_3$) and [^{3}H]inositol tetrakisphosphate ([^{3}H]$InsP_4$) were eluted by sequential addition of 0.2 M ammonium formate/0.1 M formic acid (12 ml), 0.4 M ammonium formate/0.1 M formic acid (12 ml), 0.8 M ammonium formate/0.1 M formic acid (16 ml), and 1.4 M ammonium formate/0.1 M formic acid (12 ml). These chromatographic conditions were confirmed using standards (C. Rego and E.P. Duarte, unpublished observations). The radioactivity present in each fraction was determined by liquid scintillation spectrometry after addition of 6 ml of scintillation cocktail (Universol, ICN Biomedicals).

Other methods

Results are presented as means ± SEM of the number of experiments indicated. Statistical significance was determined using the two-tailed Student's *t*-test.

RESULTS

Stimulation of cultured chick retina cells with L-glutamate (100 μM) in Na^+ medium evoked a sustained increase of the $[Ca^{2+}]_i$ by 142.4 ± 2.6 nM (Fig. 1), and this effect was not observed when Ca^{2+} was omitted from the extracellular medium (not shown). At 100 μM glutamate stimulated maximally the $[Ca^{2+}]_i$ response (not shown) and the release of [^{3}H]GABA (Hofmann & Möckel, 1991). Activation of glutamate receptors depolarizes the cells in addition to increasing Ca^{2+} conductances through the receptor associated channels

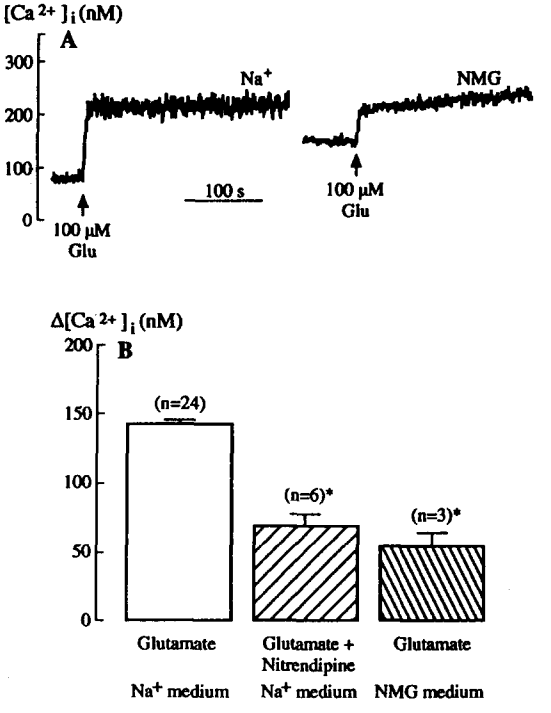

FIGURE 1. $[Ca^{2+}]_i$ responses to glutamate in Na^+ or NMG medium. The cells were loaded with Indo-1 in HEPES-buffered BME, as described in Materials and Methods, and were transferred to Na^+ (Na^+) or Mg^{2+}-free NMG media. Where indicated, 100 μM glutamate (free acid) was added (A). In (B) the bars represent average initial $[Ca^{2+}]_i$ responses ($\Delta[Ca^{2+}]_i$) to 100 μM glutamate, in NMG medium, or in Na^+-medium in the presence or in the absence of 1 μM nitrendipine. Pre-incubation with the DHP was 4 min. Results are means ± SEM for the number of experiments indicated. *Significantly different from control, $P < 0.05$.

(Ascher & Nowak, 1987; Müller *et al.*, 1992a,b; Zorumski & Thio, 1992; Burnashev *et al.*, 1992a,b), and therefore, the observed $[Ca^{2+}]_i$ responses are likely to be a combination of Ca^{2+} entry through the glutamate receptor-associated channels and through VSCCs activated as a consequence of depolarization. In order to evaluate the contribution of the receptor-associated channels to the $[Ca^{2+}]_i$ response to glutamate, the cells were stimulated in Mg^{2+}-free NMG medium (Iino *et al.*, 1990; Segal & Manor, 1992; Brorson *et al.*, 1992). Under these conditions, which allow the activation of the NMDA- and non-NMDA receptors, the effect of glutamate was inhibited to about 38% of the control, suggesting that a significant part of the response was due to Ca^{2+} entry through the receptor-associated channels. Nitrendipine (1 μM), a DHP-antagonist of the L-type VSCCs (McCleskey *et al.*, 1986) also decreased significantly (about 53%) the $[Ca^{2+}]_i$ response to glutamate. Thus, both VSCCs and the glutamate receptor-associated channels play an important role on the observed effects of glutamate on the $[Ca^{2+}]_i$.

Effect of NMDA on the $[Ca^{2+}]_i$

The mechanisms of $[Ca^{2+}]_i$ regulation by glutamate receptors were further evaluated by using specific

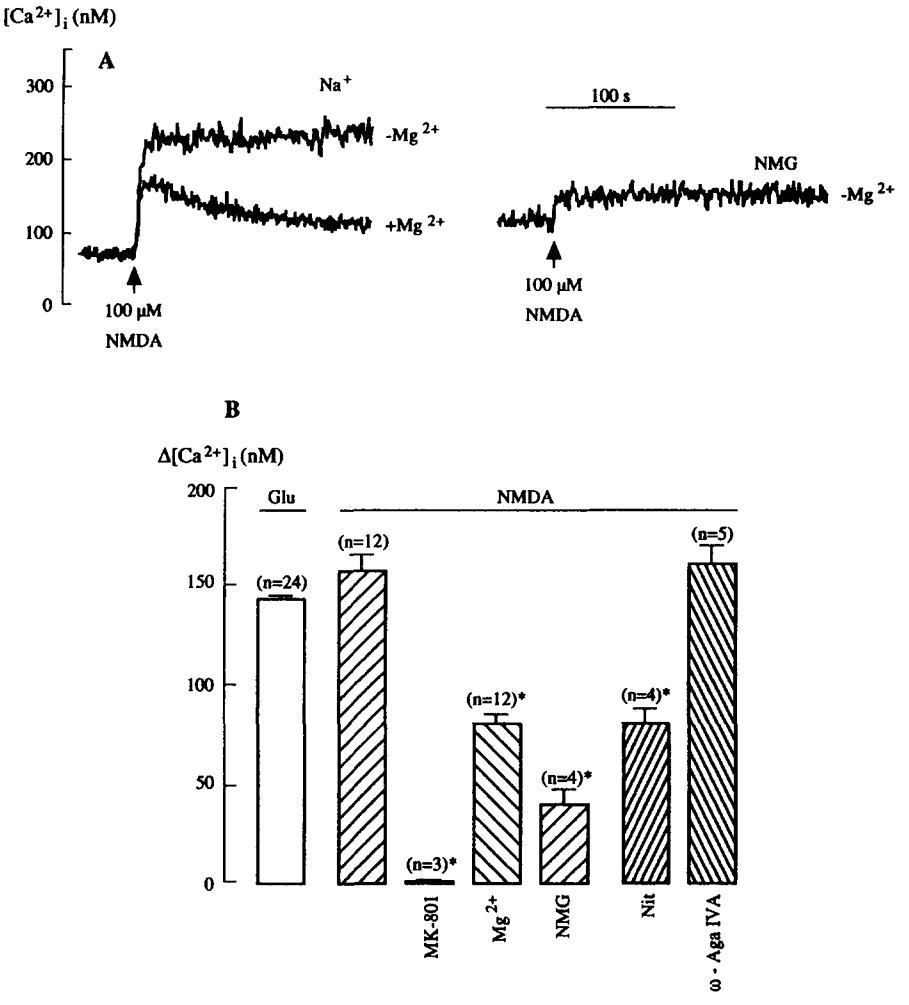

FIGURE 2. Effect of Ca^{2+} channel antagonists, MK-801 or Mg^{2+} on the $[Ca^{2+}]_i$ responses to NMDA. (A) The cells were pre-incubated for 4 min in Na^+-medium (Na^+), with ($+Mg^{2+}$) or without ($-Mg^{2+}$) 1.4 mM $MgCl_2$, or in Mg^{2+}-free NMG medium (right trace), before being stimulated with 100 μM NMDA. (B) The average initial $[Ca^{2+}]_i$ response ($\Delta[Ca^{2+}]_i$) to 100 μM NMDA in Na^+-medium, with ($+Mg^{2+}$) or without 1.4 mM $MgCl_2$, or in Mg^{2+}-free NMG medium (NMG) was compared with the effect of 100 μM glutamate (Glu), determined as indicated in Fig. 1. The effects of nitrendipine (Nit, 1.5 μM), MK-801 (7.5 μM) and ω-Aga IVA (200 nM) were tested in Mg^{2+}-free Na^+ medium, and the pre-incubation time with the antagonists was 4 min. When ω-Aga IVA was tested, the cells were also pre-incubated with the toxin for 1 hr, during the loading with Indo-1. Results are expressed as means ± SEM of the indicated number of experiments, performed in different preparations. *Significantly different from control, $P < 0.05$.

agonists of the different glutamate receptors, distinct ionic media and blockers of L-, P- and Q-type VSCCs. Thus, the non-permeable cation NMG was used to assess the contribution of each receptor-associated channel for the $[Ca^{2+}]_i$ responses, since no significant membrane depolarization and activation of VSCCs is expected to occur in the absence of extracellular Na^+. Nitrendipine (McCleskey *et al.*, 1986) was used to estimate the contribution of L-type VSCCs and the toxin ω-Aga IVA (Mintz *et al.*, 1992; Wheeler *et al.*, 1994) was utilized to determine the role of P- and Q-type VSCCs to the observed $[Ca^{2+}]_i$ responses. At 1 μM nitrendipine inhibited maximally and specifically the $[Ca^{2+}]_i$ transient due to 25 mM KCl-depolarization ($\Delta[Ca^{2+}]_i$ = 470.5 ± 10.0 nM; $n = 5$) by 39.9 ± 1.6% ($n = 5$), and therefore this concentration was used as a probe for the role of L-type VSCCs. The effect of antagonists of the N-type VSCCs was not tested since we have shown before that ω-conotoxin GVIA is without effect on the $[Ca^{2+}]_i$ responses evoked by glutamate in cultured chick retina cells (Duarte *et al.*, 1993).

In Mg^{2+}-free Na^+ medium, 100 μM NMDA evoked a sustained increase of the $[Ca^{2+}]_i$ of 156.6 ± 8.2 nM, and this effect was completely reversed by 7.5 μM MK-801 (Fig. 2), a non-competitive antagonist of the NMDA receptors (Wong *et al.*, 1986). After stimulation with 100 μM NMDA, the subsequent addition of 10 μM glycine or NMDA was without effect (not shown), indicating that saturating concentrations of glutamate and the maximal activation of the NMDA receptors have similar effects on the $[Ca^{2+}]_i$ ($P = 0.045$). In the presence of 1.4 mM $MgCl_2$, NMDA increased rapidly the $[Ca^{2+}]_i$ by 77.1 ± 4.6 nM, but after the initial peak the $[Ca^{2+}]_i$ decreased towards a plateau 32.3 ± 5.2 nM above the resting concentration [Fig. 2(A)].

In order to test for the role of L-, P- and Q-type of

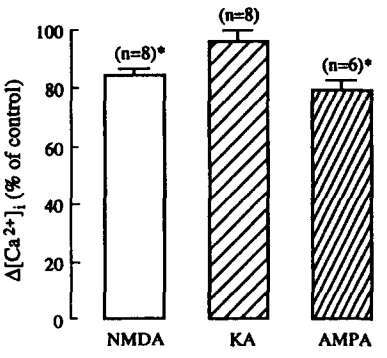

FIGURE 3. Effect of thapsigargin on the initial $[Ca^{2+}]_i$ response to NMDA, kainate and AMPA. The cells were pre-incubated with 1–5 μM thapsigargin for 3–4 min, and thereafter stimulated with 100 μM NMDA, 100 μM kainate (KA) or 100 μM AMPA. The initial $[Ca^{2+}]_i$ response was compared with that observed under control conditions, in the same preparation. The results are expressed as means ± SEM of the indicated number of experiments, performed in different preparations. *Significantly different from control, $P < 0.05$.

VSCCs on the $[Ca^{2+}]_i$ changes evoked by NMDA, we studied the effect of specific antagonists in Mg^{2+}-free Na^+ medium. Pre-incubation of the cells with 1.5 μM nitrendipine inhibited the response to 100 μM NMDA by 51%, whereas 200 nM ω-Aga IVA was without effect (Fig. 2). The effect of the DHP nifedipine (1.5 μM) was similar to that observed in the presence of nitrendipine (not shown). When the extracellular Na^+ was iso-osmotically replaced by NMG, and in the absence of Mg^{2+}, the response to NMDA was decreased to 39.8 ± 7.4 nM, corresponding to the contribution of the NMDA receptor-associated channels to the $[Ca^{2+}]_i$ responses. The lower effect of NMDA in NMG medium than in Na^+ medium is not directly due to the absence of Na^+, because the cation has been shown to inhibit the binding of [^{3}H]glutamate to the NMDA recognition site on the receptor–ionophore complex (Yoneda & Ogita, 1991).

The $[Ca^{2+}]_i$ response to NMDA may involve a component due to CICR (Kostyuk & Verkhratsky, 1994). Therefore, experiments were performed using thapsigargin, which depletes non-mitochondrial Ca^{2+} stores in a wide variety of cells by inhibiting microsomal Ca^{2+} ATPases (Thastrup *et al.*, 1990). In cultured chick retina cells, 1–5 μM thapsigargin raised transiently the $[Ca^{2+}]_i$ by 64.8 ± 2.6 nM ($n = 36$). After depletion of the thapsigargin-sensitive Ca^{2+} stores, the shape of the $[Ca^{2+}]_i$ response to NMDA was not affected (not shown), but the magnitude was significantly decreased ($P < 0.05$) to 84.0 ± 2.1% ($n = 8$) of the control (Fig. 3).

$[Ca^{2+}]_i$ responses to kainate

Addition of 100 μM kainate to cultured chick retina cells in Na^+ medium rapidly increased the $[Ca^{2+}]_i$ by 276.5 ± 6.8 nM (Fig. 4), and the $[Ca^{2+}]_i$ decreased afterwards to a plateau which was 74.0 ± 2.1% of the initial increment. CNQX (20 μM), a non-selective blocker of the non-NMDA glutamate receptors (Watkins *et al.*, 1990), decreased the initial $[Ca^{2+}]_i$ rise to 66.3 ± 7.2 nM. Dose–response curves up to 30 μM showed that 10 μM CNQX already produced maximal inhibition (not shown). This effect was not due to a non-specific interaction with the glycine site at the NMDA receptor, since 10 μM 5,7-DCK did not inhibit ($P > 0.05$) the $[Ca^{2+}]_i$ response to kainate (99.2 ± 3.4%; $n = 3$). When the extracellular Na^+ was iso-osmotically replaced by NMG, kainate evoked a sustained increase of the $[Ca^{2+}]_i$, which was about 60% of the control (Fig. 4). As expected, this response was not significantly affected ($P > 0.05$) by 1.5 μM nifendipine (105.5 ± 5.2%; $n = 4$). These results suggest that the transient component of the response is due to Ca^{2+} entry through VSCCs, and that the kainate receptor-associated channel also allows the entry of Ca^{2+} [Fig. 4(A)].

Figure 4(B) shows the effect of pre-incubating the cells with nitrendipine or ω-Aga IVA on the initial increase of the kainate-induced $[Ca^{2+}]_i$ response. A representative example of the effect of ω-Aga IVA is also shown in Fig. 4(A). When the cells were pre-incubated with the toxin, the initial $[Ca^{2+}]_i$ response decreased to 75.5% of the control [Fig. 4(B)], mainly due to the effect on the transient component [cf. Fig. 4(A) left (Na^+) and right traces]. Nitrendipine decreased the initial $[Ca^{2+}]_i$ response to kainate to 225.3 ± 14.8 nM, and a similar inhibition was observed on the plateau phase (from 230.6 ± 10.3 to 181.4 ± 11.2 nM).

In order to test for the role of Ca^{2+} stored in intracellular non-mitochondrial compartments to the $[Ca^{2+}]_i$ transients evoked by kainate, the cells were pre-incubated with 1–5 μM thapsigargin for 3–4 min before stimulation with the agonist. After depletion of the Ca^{2+} stores the initial $[Ca^{2+}]_i$ rise was not significanty affected (Fig. 3; 95.7 ± 3.5% of control) ($P > 0.05$). Moreover, the steady-state $[Ca^{2+}]_i$ plateau in the presence of thapsigargin (69.8 ± 2.7% of the initial $[Ca^{2+}]_i$ increment) was not significantly different from that determined under control conditions ($P > 0.05$).

Effect of AMPA on the $[Ca^{2+}]_i$

The maximal activation of the AMPA receptors, observed with 100 μM AMPA (not shown), increased transiently the $[Ca^{2+}]_i$ by 120.4 ± 6.0 nM, which is significantly lower ($P < 0.05$) than that induced by glutamate (Fig. 5). After the initial rapid rise, the $[Ca^{2+}]_i$ decreased slowly towards a plateau 87.0 ± 9.6 nM above the resting concentration [Fig. 5(A)]. The effect of AMPA was only slightly affected by 10–30 μM CNQX (10% inhibition), but was strongly depressed in Na^+-free NMG medium (12.5% of the control). As expected, the small effect of CNQX was not due to a non-specific binding to the glycine site of the NMDA receptor, since 10 μM 5,7-DCK did not inhibit ($P > 0.05$) the $[Ca^{2+}]_i$ response to AMPA (99.1 ± 1.3%; $n = 3$). The importance of the VSCCs to the $[Ca^{2+}]_i$ response to AMPA was further emphasized by the 54% inhibition observed after pre-incubation of the cells with nitrendipine (Fig. 5). The DHP also decreased the plateau phase of the $[Ca^{2+}]_i$ response to 43.1 ± 5.1 nM. Similarly

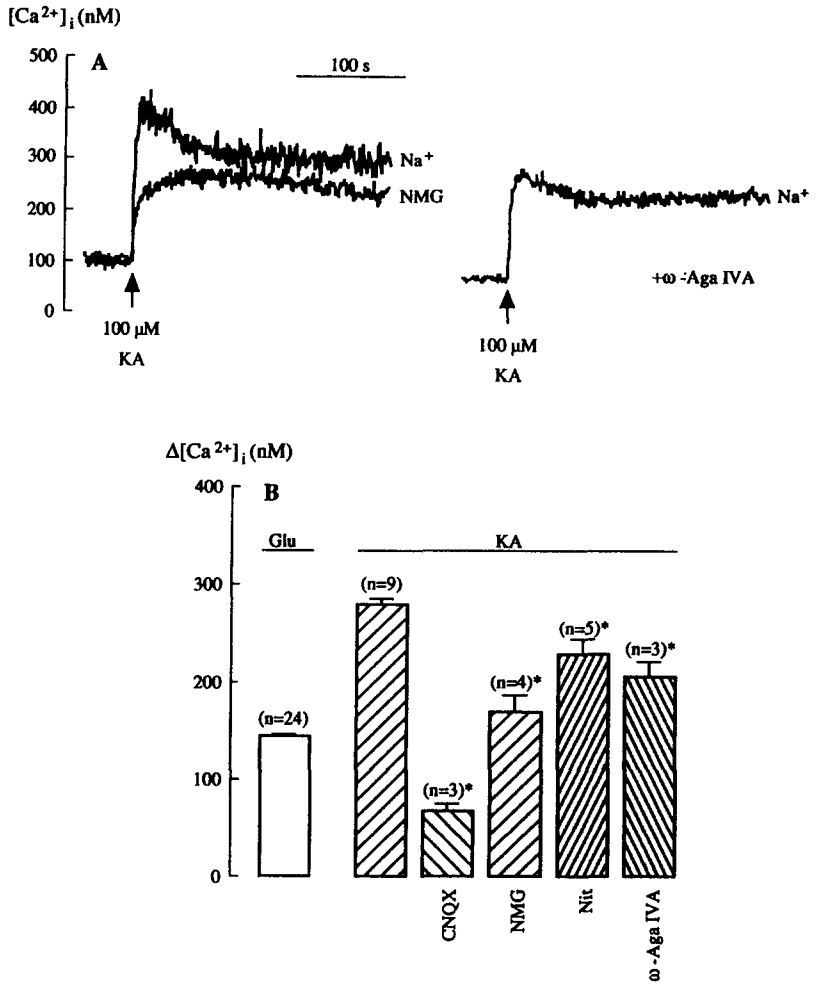

FIGURE 4. Effect of CNQX and Ca^{2+} channel antagonists on the $[Ca^{2+}]_i$ responses to kainate. In (A) the cells were pre-incubated for 4 min in Na^+ medium (Na^+) or in NMG medium (NMG), and, where indicated, 100 μM kainate (KA) was added. When the effect of 200 nM ω-Aga IVA was tested (right trace), the cells were also pre-incubated with the toxin for 1 hr, during the loading with Indo-1. (B) The magnitude of the initial $[Ca^{2+}]_i$ response ($\Delta[Ca^{2+}]_i$) to 100 μM kainate (KA) in Na^+ or in NMG media was compared with the effect of 100 μM glutamate (Glu) in Na^+ medium (see Fig. 1). The effects of nitrendipine (Nit, 1.5 μM), CNQX (20 μM) or 200 nM ω-Aga IVA on the response to kainate were tested after 4 min pre-incubation with the antagonists. When ω-Aga IVA was tested the toxin was also added to the Indo-1 loading medium. Results are expressed as means ± SEM of the indicated number of experiments, carried out in different preparartions. *Significantly different from control, $P < 0.05$.

to NMDA, the effects of AMPA on the $[Ca^{2+}]_i$ were not affected by ω-Aga IVA. Moreover, depletion of the thapsigargin-sensitive intracellular Ca^{2+} stores decreased the initial AMPA-induced $[Ca^{2+}]_i$ increase to 79.0 ± 3.3% of the control (Fig. 3), but did not affect the rate of recovery (not shown).

1S,1R-ACPD stimulates inositol phospholipid hydrolysis, but does not affect the $[Ca^{2+}]_i$

Glutamate may also increase the $[Ca^{2+}]_i$ by stimulating the inositol phospholipid cycle, and thus mobilizing intracellular Ca^{2+}, via the metabotropic ACPD receptors (Sladeczek *et al.*, 1985; Osborne, 1990). 1*S*,3*R*-ACPD (200 μM) did not affect the $[Ca^{2+}]_i$ in cultured chick retina cells, in the absence or in the presence of extracellular Ca^{2+} (Fig. 6). In Ca^{2+}-free medium (1 mM EGTA) the subsequent addition of 3 μM ionomycin increased transiently the $[Ca^{2+}]_i$ by 82.4 ± 1.6 nM ($n = 3$), most likely by mobilizing intracellular Ca^{2+}.

The presence of ACPD receptors in chick retina cells was further evaluated by measuring the accumulation of myo-[^{3}H]inositol phosphates evoked by 2 min stimulation with 1*S*,3*R*-ACPD (200 μM). The agonist increased significantly ($P < 0.05$) the accumulation of [^{3}H]$InsP_1$, [^{3}H]$InsP_2$ and [^{3}H]$InsP_3$, but this effect was not antagonized by L-AP3 (100–500 μM), a rather selective antagonist of some of the metabotropic glutamate receptors (Schoepp & Conn, 1993). Glutamate (100 μM) had similar effects ($P > 0.05$) on the accumulation of [^{3}H]$InsP_1$ and [^{3}H]$InsP_3$, but was more effective ($P < 0.05$) in stimulating the production of [^{3}H]$InsP_2$ and [^{3}H]$InsP_4$. As the retinal cultures used for these studies contain a significant percentage of glial-like flat cells (Adler *et al.*, 1982), it was of interest to ascertain their

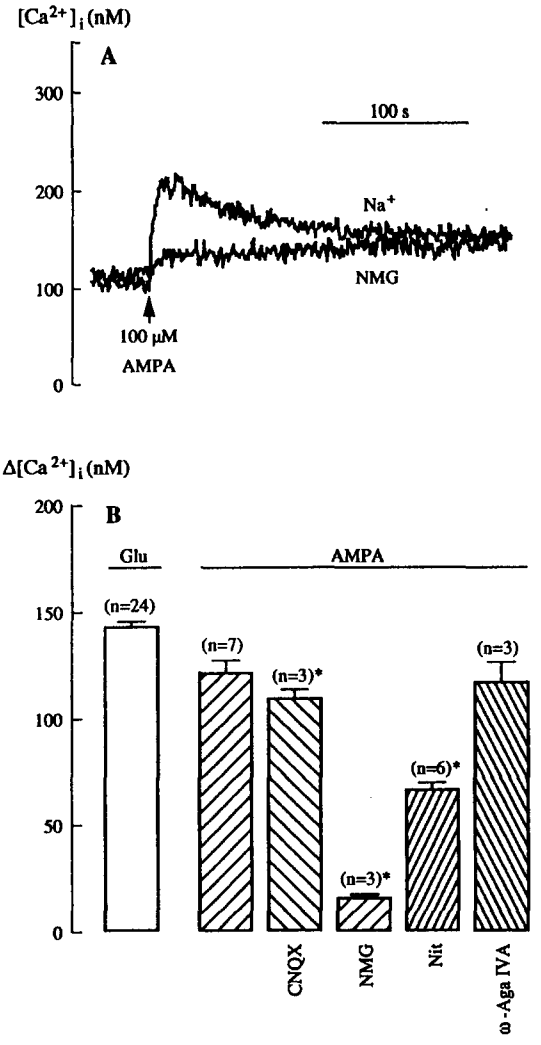

FIGURE 5. Effect of CNQX and Ca^{2+} channel antagonists on the $[Ca^{2+}]_i$ response to AMPA. (A) Representative traces of the effect of 100 μM AMPA on the $[Ca^{2+}]_i$, in Na^+ or NMG media. (B) Average initial $[Ca^{2+}]_i$ responses ($\Delta[Ca^{2+}]_i$) to 100 μM AMPA in Na^+ or NMG media, as compared with the effect of 100 μM glutamate (Glu) in Na^+ medium (see Fig. 1). The pre-incubation time with nitrendipine (Nit, 1.5 μM), CNQX (20 μM) or ω-Aga IVA (200 nM) was 4 min. When the effect of the toxin was tested it was also present during the loading with Indo-1. Results are expressed as means ± SEM of the indicated number of experiments, performed in different preparations. *Significantly different from control, $P < 0.05$.

contribution, if any, to the effect of 1*S*,3*R*-ACPD on the phosphoinositide breakdown. The stimulation of purified cultures of flat cells with 1*S*,3*R*-ACPD did not stimulate the production of [^{3}H]inositol phosphates (Fig. 7, rightmost bars). Thus, the 1*S*,3*R*-ACPD-induced inositol phosphate formation in mixed neuronal and glial cultures is due to the activation of retinal neurons.

DISCUSSION

Glutamate is probably the main light-evoked excitatory neurotransmitter activating amacrine cells (Linn & Massey, 1991; Linn *et al.*, 1991; Barnstable, 1993). The present results show that the agonists of the ionotropic glutamate receptors NMDA, kainate and AMPA increase the $[Ca^{2+}]_i$ in cultures enriched in chick retinal amacrine-like cells. In all cases the Ca^{2+} signals were found to be composite responses comprising Ca^{2+} permeating the receptor-associated channels and Ca^{2+} entering through voltage-gated Ca^{2+} channels. Furthermore, the $[Ca^{2+}]_i$ responses to NMDA and AMPA also involved a magnification factor provided by Ca^{2+} release from intracellular stores.

Basic properties of the ionotropic glutamate receptors

According to the well known pharmacology of the glutamate receptors, the effects of NMDA and kainate were significantly inhibited by MK-801 and CNQX, respectively. However, to our surprise, the $[Ca^{2+}]_i$ increase due to activation of the AMPA receptors was largely insensitive to CNQX, even at high concentrations of the antagonist (Fig. 5). This is not due to the degradation of the antagonist because it inhibited significantly the effect of kainate.

Also surprising was the observation that NMDA could elicit a transient increase of the $[Ca^{2+}]_i$, even in the presence of 1.4 mM $MgCl_2$ [Fig. 2(A)]. This observation indicates either that (1) in the cultured retinal neurons the NMDA receptors are not sensitive to voltage-dependent channel blockade by Mg^{2+}, or that (2) the cells are partially depolarized, possibly due to the action of endogenous neurotransmitters. The modulation of the NMDA receptor by endogenous substances is suggested by the observation that the addition of exogenous glycine, a co-agonist at the NMDA receptor–channel complex (Zorumski & Thio, 1992), did not affect the $[Ca^{2+}]_i$ response to the agonist. However, NMDA receptors with low sensitivity to Mg^{2+} have also been described in different cell types (e.g. Kato & Yoshimura, 1993).

Ca^{2+} *entry through glutamate receptors*

In the hippocampus the replacement of Na^+ by NMG was shown to shift the reversal of the NMDA-induced currents in the hyperpolarizing direction, and prevents the depolarization-induced activation of VSCCs (Iino *et al.*, 1990; Segal & Manor, 1992). Accordingly, in this work the permeation of Ca^{2+} through the glutamate receptor-associated channels was determined by stimulating the cells in Na^+-free NMG medium. We have measured significant increases in the intracellular Ca^{2+} concentration due to Ca^{2+} influx through NMDA (Fig. 2), as well as non-NMDA (Fig. 4 and Fig. 5) gated channels. These results differ from our previous report where we were unable to resolve the Ca^{2+} entry through the glutamate receptor-associated channels, probably because the number of cells utilized in that study was very small and did not permit detection of small changes in the $[Ca^{2+}]_i$ (Duarte *et al.*, 1993).

The $[Ca^{2+}]_i$ increase due to Ca^{2+} entry through the NMDA receptor-associated channel, as determined in Mg^{2+}-free NMG medium, was about 25% of the total response to the agonist in Na^+ medium. Calcium permeation through the NMDA receptor-associated channels has been reported in different cell types, based on single-channel currents (Ascher & Nowak, 1987), ion

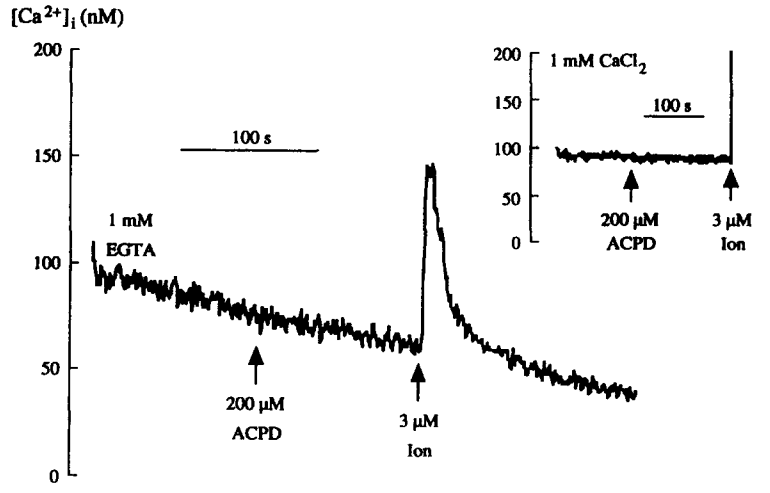

FIGURE 6. Lack of effect of ACPD on the $[Ca^{2+}]_i$. The cells were pre-incubated for 2 min in Ca^{2+}-free (1 mM EGTA; main figure) or in Ca^{2+}-containing (1 mM $CaCl_2$) Na^+ medium, and where indicated 200 μM ACPD was added. Traces are representative of three experiments performed in independent preparations.

substitution experiments (Iino *et al.*, 1990), on shifts in the reversal potentials that occur when extracellular Ca^{2+} concentrations are increased and using Ca^{2+}-sensitive fluorescent dyes (e.g. Mayer & Westbrook, 1987; Segal & Manor, 1992; Schneggenburger *et al.*, 1993).

In most neurons that have been studied, the non-NMDA ionotropic glutamate receptors exhibit low Ca^{2+} permeability (Mayer & Westbrook, 1987), although in some neurons and glial cells it may be considerably higher (e.g. Gilbertson *et al.*, 1991; Brorson *et al.*, 1992; Burnashev *et al.*, 1992a,b; Segal & Manor, 1992; Schneggenburger *et al.*, 1993). In NMG medium, the $[Ca^{2+}]_i$ increase due to Ca^{2+} entry through the kainate receptor-associated channel was higher than that observed upon activation of the NMDA receptor (Fig. 2 and Fig. 4). The influx of Ca^{2+} through the kainate receptor corresponds to about 60% of the initial $[Ca^{2+}]_i$ response to kainate. Although the removal of $[Na^+]_o$ may lead to the accumulation of calcium inside the cells upon activation of the kainate receptors (Segal & Manor, 1992), the contribution of the receptor-associated channels is probably not overestimated because the Na^+/Ca^{2+} exchanger is not expected to participate significantly on the early phases of the $[Ca^{2+}]_i$ response (Segal & Manor, 1992). The Ca^{2+} permeability of the AMPA receptors in the cultured chick retina cells was very low, being responsible for only 12.5% of the $[Ca^{2+}]_i$ response determined in Na^+ medium.

Considering that the retinal non-NMDA receptors probably are oligomeric assemblies of subunits, different combinations of the isoforms may generate subspecies of receptors with different selectivities for permeating ions. Although it is still not known whether the isoforms assemble randomly to form functional receptors, the Ca^{2+} permeable kainate receptor described here, and the Ca^{2+}-impermeable non-NMDA receptors reported in other systems (Mayer & Westbrook, 1987; Brorson *et al.*, 1992; Iino *et al.*, 1990; Segal & Manor, 1992; Reichling & MacDermott, 1993), may result from the functional polymorphism. Expression studies have shown that GluR1–GluR7 subunits are present in the inner nuclear layer of the rat retina, where the amacrine cells are localized (Müller *et al.*, 1992; Hamassaki-Britto *et al.*, 1993).

Ca^{2+} *entry through voltage-sensitive* Ca^{2+} *channels*

The cultured chick retina cells are equipped with at least three pharmacologically distinct classes of VSCCs: 1,4-dihydropyridine-sensitive L-type channels (Wei *et al.*, 1989; Duarte *et al.*, 1992), ω-CgTx-sensitive N-type channels, and those insensitive to the above mentioned antagonists (Duarte *et al.*, 1992, 1993). In this work, we show that 200 nM ω-Aga IVA decreases partially the $[Ca^{2+}]_i$ response to kainate (Fig. 4). At that concentration the toxin fully blocks the P-type VSCCs, whereas the Q-type channels are only partially inhibited (Sather *et al.*, 1993). Therefore, our results indicate that the P- and/or Q-type Ca^{2+} channels are activated upon stimulation of the kainate, but not NMDA or AMPA receptors. The P channels are also present in the horizontal cells of the retina, as demonstrated electrophysiologically in the white bass retina (Sullivan & Lasater, 1992).

The DHP nitrendipine inhibited the response to glutamate by 48%, suggesting that the L-type VSCCs play a very important role on the $[Ca^{2+}]_i$ transients generated upon activation of the glutamate receptors. Indeed, nitrendipine decreased by 76, 51, and 55 nM the $[Ca^{2+}]_i$ increase evoked by NMDA, kainate and AMPA, respectively. The effect of the DHP on the response to NMDA may be overestimated due to the reported direct action of nitrendipine, but not nifedipine, on the NMDA receptor–channel complex (Skeen *et al.*, 1993). However, the observation that nifedipine inhibits to the same extent as nitrendipine the $[Ca^{2+}]_i$ response to NMDA (not shown) ruled out the contribution of non-specific side effects of nitrendipine under our experimental conditions.

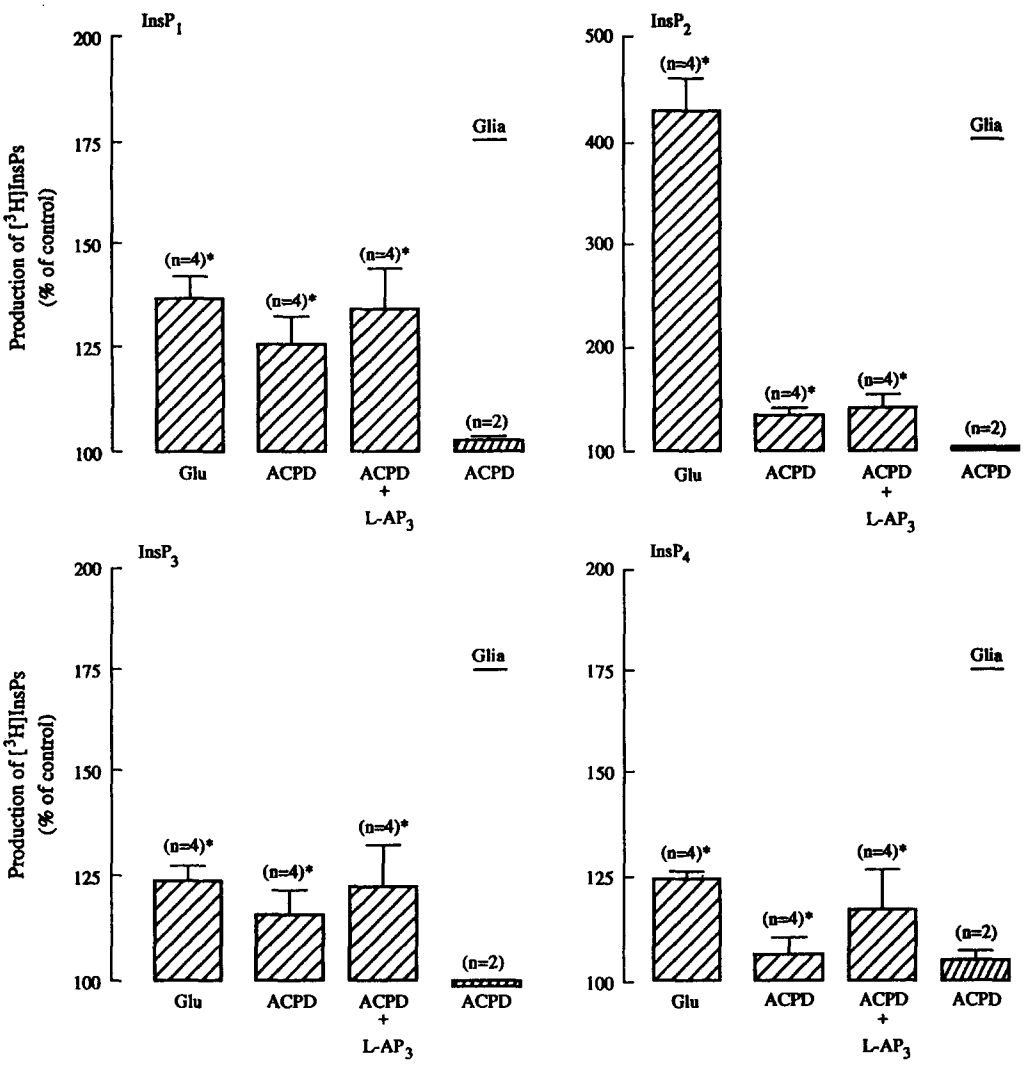

FIGURE 7. Effect of glutamate and 1*S*,3*R*-ACPD on the accumulation of [^{3}H]inositol phosphates in cultures enriched in retinal neurons or cultured glial cells (Glia). The cells were labeled with [^{3}H]inositol as described in Materials and Methods, and were incubated for 2 min with 100 μM glutamate (Glu) or 200 μM 1*S*,3*R*-ACPD (ACPD). When L-AP3 was tested the cells were pre-incubated with the antagonist for 10 min, during the incubation in 10 mM Li^+ buffer. Results are expressed as a percentage of the control values, determined in Na^+ medium, and represent means ± SEM of triplicate determinations performed in the indicated number of preparations. The control values for the cultures containing retinal neurons were (in DPM × 10^{-3}/mg protein): IP_1, 125.7 ± 10.9; IP_2, 24.6 ± 2.6; IP_3, 15.6 ± 1.2; IP_4, 11.3 ± 1.8. For the glial cell cultures the following control values were obtained (in DPM × 10^{-3}/mg protein): IP_1, 161.0 ± 19.4; IP_2, 29.1 ± 4.5; IP_3, 24.5 ± 2.1; IP_4, 12.0 ± 1.4. *Significantly different from the basal production of [^{3}H]InsPs, $P < 0.05$.

The P-type Ca^{2+} channels also contribute significantly to the response to kainate, but ω-Aga IVA was without effect on the $[Ca^{2+}]_i$ transients evoked by NMDA and AMPA. Moreover, it is interesting to note that although the N-type VSCCs are present in our cultures (Duarte *et al.*, 1992) they do not contribute to the $[Ca^{2+}]_i$ responses to glutamate (Duarte *et al.*, 1993). The differential activation of VSCCs by the NMDA, kainate and AMPA receptors may reflect that (1) the receptors are localized in different cells, which may or may not contain P- and/or Q-type Ca^{2+} channels, being the L-type Ca^{2+} channels always present; or (2) that the receptors and the P and/or Q-type Ca^{2+} channels may have a heterogeneous distribution within the cells. The former hypothesis is favoured by the identification, in the tiger salamander retina, of subpopulations of amacrine cells displaying light-evoked responses due to activation of NMDA- and non-NMDA receptors, whereas the response of other cells is mediated only by non-NMDA receptors (Dixon & Copenhagen, 1992). Similarly, the effect of glutamate on the rat AII-amacrine cells is due to the activation of kainate and AMPA receptors, whereas NMDA is without effect (Boos *et al.*, 1993).

Release of Ca^{2+} *from intracellular stores*

1*S*,3*R*-ACPD increased the intracellular accumulation of myo-[^{3}H]inositol phosphates, but was without effect on the $[Ca^{2+}]_i$, in Ca^{2+}-containing or Ca^{2+}-free media (Figs 6 and 7). Our results are similar to those reported in other preparations (e.g. Linn & Christensen, 1992), but

contrast with observations in cultured rabbit retina cells where ibotenate was shown to increase the $[Ca^{2+}]_i$ by stimulating the hydrolysis of phosphoinositides (Osborne, 1990). Glutamate was much more efficient than ACPD in stimulating the accumulation of $InsP_2$. This difference may rely on the $[Ca^{2+}]_i$ rise evoked by glutamate, which is expected to activate directly phospholipase C (Eberhard & Holz, 1988).

Molecular biology studies have now identified six different metabotropic receptor genes (mGluR-1–mGluR-6), which have a different distribution in the brain (Schoepp & Conn, 1993; Hollmann & Heinemann, 1994). The mGluR family has been divided into three different groups, based on the signal transduction mechanisms and on pharmacological properties. mGluR-1 and mGluR-5 are linked to the hydrolysis of phosphoinositides, but whereas L-AP3 behaves as a weak inhibitor of mGluR-1 it has no effect on mGluR-5 (Schoepp & Conn, 1993). Thus, based on the observed lack of effect of L-AP3 on the intracellular accumulation of phosphoinositides, it is tempting to suggest that the mGluR-5 type of glutamate receptors are present in the cultured chick retina cells. Since our studies were carried out in cultures containing neuronal and glial-like flat retina cells, we were concerned that the observed effects of 1*S*,3*R*-ACPD were due to the activation of the metabotropic receptors on the retinal flat cells. However, our results show that the cultured retinal glial cells do not possess metabotropic glutamate receptors coupled to the hydrolysis of phosphoinositides. Similarly, it was recently shown that primary cultures of Müller cells from chick retina are not endowed with ACPD receptors (López-Colomé, Ortega & Romo-de-Vivar, 1993).

In the salamander retina the amacrine cells show uniform $InsP_3$ receptor-like immunoreactivity through their processes, which are both presynaptic and postsynaptic in nature (Peng *et al.*, 1991). The observation that in our cultures 1*S*,3*R*-ACPD stimulates the accumulation of inositol phosphates without affecting the $[Ca^{2+}]_i$, suggests that the important signal may be diacylglycerol, the other second messenger produced by the phosphoinositides turnover. In the brain it has been suggested that one or the other of the two branches of this signalling pathway may be biased in a given location (Worley *et al.*, 1987).

The Ca^{2+}-induced Ca^{2+} release was found to participate in the magnification of the $[Ca^{2+}]_i$ responses triggered by NMDA (Segal & Manor, 1992; Simpson *et al.*, 1993) and kainate (Linn & Christensen, 1992; Kocsis *et al.*, 1993). In cultured chick retina cells the inhibitor of the microsomal Ca^{2+} ATPases (Thastrup *et al.*, 1990) raised transiently the $[Ca^{2+}]_i$, and decreased the initial $[Ca^{2+}]_i$ increase stimulated by NMDA and AMPA by about 15 and 21%, respectively. However, the depletion of the intracellular Ca^{2+} stores did not affect the shape of the $[Ca^{2+}]_i$ responses. The results suggest that CICR may be also an important component of the responses to NMDA and AMPA. It was recently shown in adrenal glomerulosa cells that thapsigargin inhibits L-type Ca^{2+} channels (Rossie *et al.*, 1993). However, the lack of effect of thapsigargin on the $[Ca^{2+}]_i$ response to kainate, which is also partially mediated by L-type Ca^{2+} channels, suggests that the results reported here are specifically due to the depletion of intracellular Ca^{2+} stores.

It was surprising that kainate, the most potent agonist in increasing the $[Ca^{2+}]_i$, was unable to trigger CICR. Our results are also in apparent contradiction with the observed reduction by caffeine of the kainate-induced $[Ca^{2+}]_i$ response in retina neurons (Kocsis *et al.*, 1993). However, this difference may indicate that in the cultured retinal neurons thapsigargin and caffeine do not act on identical pools of Ca^{2+}. Taken together our results suggest that in the retinal neurons the kainate receptors (but not the AMPA and NMDA receptors) (1) are localized far from the thapsigargin-sensitive intracellular stores and/or (2) are not as efficient as the NMDA and AMPA receptors in activating the enzyme responsible for the synthesis of cADPribose. This compound has been proposed as an endogenous regulator of CICR through a ryanodine receptor (Kostyuk & Verkhratsky, 1994).

CONCLUSIONS

The results show that the $[Ca^{2+}]_i$ responses to stimulation of the ionotropic glutamate receptors are mediated by different mechanisms: influx of Ca^{2+} through the receptor-associated channels, which is most significant upon stimulation with kainate, and depolarization-induced activation of VSCCs. Moreover, activation of the AMPA and NMDA receptors also evoke Ca^{2+} release from thapsigargin-sensitive Ca^{2+} stores. The maximal $[Ca^{2+}]_i$ changes occurred when the cells were stimulated with kainate, followed by NMDA and AMPA. This may reflect that (1) there are relatively more kainate than NMDA and AMPA receptors and/or this may be due to (2) the high Ca^{2+} permeability of the kainate receptor-associated channels.

The influx of Ca^{2+} through the L-type VSCCs may be involved in the regulation of neurotransmitter release. Indeed, we have shown that in cultured chick retina cells the nitrendipine-sensitive Ca^{2+} channels regulate the exocytotic release of [^{3}H]GABA (Duarte *et al.*, 1992). The functional significance of the high Ca^{2+}-permeability of the kainate receptor-associated channels is not so clear. This Ca^{2+} permeable pathway may be of great significance in mediating excitatory amino acid induced phenomena of various types, particularly when the NMDA receptors are not present (Dixon & Copenhagen, 1992). Moreover, the influx of Ca^{2+} through the kainate receptor is assumed to contribute to the kainate-induced neurotoxicity in some neurons *in vitro* (Murphy & Miller, 1989). In cultured retina cells, the entry of Ca^{2+} through the domoic acid activated kainate receptor was shown to trigger the exocytotic release of [^{3}H]GABA (Alfonso *et al.*, 1994).

We have also shown that part of the $[Ca^{2+}]_i$ responses observed upon activation of the AMPA and NMDA receptors were due to Ca^{2+} release from intracellular stores, possibly by CICR. The Ca^{2+} stored in intracellular

compartments may play an important role in the glutamate-induced neuronal degeneration (Frandsen & Schousboe, 1991).

Other mechanisms have been proposed to contribute to the $[Ca^{2+}]_i$ responses to glutamate: stretch-activated conductances, leak conductances activated by cells swelling and the Na^+/Ca^{2+} exchanger (Choi, 1988). The latter mechanism has been shown to affect the $[Ca^{2+}]_i$ in nerve terminals isolated from the brain cortex (Duarte *et al.*, 1991). In our cultures the activation of the metabotropic glutamate receptors did not affect the $[Ca^{2+}]_i$, but stimulated the intracellular accumulation of inositol phosphates. In cerebrocortical synaptosomes the diacylglycerol generated by this signalling pathway regulates the exocytotic release of glutamate (Herrero *et al.*, 1992).

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