

Metabotropic glutamate and dopamine receptors co-regulate AMPA receptor activity through PKA in cultured chick retinal neurones: effect on GluR4 phosphorylation and surface expression

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Abstract

Glutamate receptor phosphorylation has been implicated in several forms of modulation of synaptic transmission. It has been reported that protein kinase A (PKA) can phosphorylate the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor subunit GluR4 on Ser842, both *in vitro* and *in vivo*. Here, we studied the regulation of GluR4 phosphorylation and intracellular trafficking by PKA and by metabotropic receptors coupled to adenylyl cyclase (AC), in cultured chick retinal amacrine-like neurones, which are enriched in GluR4. The regulation of AMPA receptor activity by PKA and by metabotropic AC-coupled receptors was also investigated by measuring the $[Ca^{2+}]_i$ response to kainate in Na^+ -free medium. Stimulation of AC with forskolin (FSK), or using the selective agonist of dopamine D₁ receptors (\pm)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol (SKF38393), increased the $[Ca^{2+}]_i$ response to

kainate, GluR4 phosphorylation at Ser842 and GluR4 surface expression. Pre-incubation of the cells with (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV), an agonist of group II metabotropic glutamate receptors (mGluR), which are coupled to inhibition of AC, inhibited the effect of FSK and of SKF38393 on AMPA receptor activity, GluR4 phosphorylation and expression at the plasma membrane. These results indicate that there is a functional cross-talk between dopamine D₁ receptors and group II mGluR in the regulation of GluR4 phosphorylation and AMPA receptor activity. Our data show that GluR4 phosphorylation at Ser842 by PKA, and its recruitment to the plasma membrane upon phosphorylation, is regulated by metabotropic receptors.

Keywords: AMPA receptors, chick retina, GluR4, metabotropic receptors, protein kinase A, surface expression.

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Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS) and its receptors have been shown to play an important role in synaptic plasticity (Bear and Abraham 1996; Malenka and Nicoll 1999), cell death (Choi 1988) and neural development (Constantine-Paton

Abbreviations used: AC, adenylyl cyclase; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate; BCA, bichoninic acid; BME, basal medium of Eagle; BSA, bovine serum albumin; CaMKII, Ca^{2+} /calmodulin-dependent protein kinase II; CHEL, chelerythrine chloride; db-cAMP, N⁶,2'-O-dibutyryl adenosine-3',5'-cyclic monophosphate, sodium salt monohydrate; DCG-IV, (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine; DOC, deoxycholic acid; FSK, forskolin; H-89, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide. 2HCl; Indo-1/AM, indo-1/acetoxymethyl ester; LTP, long-term potentiation; mGluR, metabotropic glutamate receptors; NMG, N-methyl-D-glucamine; PKA, protein kinase A; PKC, protein kinase C; PVDF, polyvinylidene difluoride; RO-20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; Rp-cAMPs, Rp-adenosine-3',5'-cyclic monophosphorothioate; SKF38393, (\pm)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol; SKF83566, (\pm)-7-bromo-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride; SDS, sodium dodecyl sulfate.

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et al. 1990). Glutamate receptors belong to the ionotropic and metabotropic families of receptors (reviewed in Ozawa *et al.* 1998). Ionotropic glutamate receptors are ligand-gated ion channels that mediate most of the rapid excitatory synaptic transmission in the CNS. These receptors are classified according to their pharmacological and electrophysiological properties as *N*-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) or kainate receptors (reviewed in Wisden and Seeburg 1993; Hollmann and Heinemann 1994). Metabotropic glutamate receptors do not form ion pores or mediate fast synaptic transmission. These receptors act via second messengers and have a modulatory role over several cellular processes, including ionotropic receptor response (Pin and Bockaert 1995).

Phosphorylation is perhaps the most common form of post-translational modification of ion channels and ionotropic receptors. In general, phosphorylation can modulate the main characteristics of the channel, including its open probability and mean open time, as well as intracellular receptor trafficking (reviewed in Carvalho *et al.* 2000; Barry and Ziff 2002; Malinow and Malenka 2002; Song and Huganir 2002; Gomes *et al.* 2003). Phosphorylation of non-NMDA receptors by protein kinase A (PKA), following stimulation of adenylyl cyclase (AC) with forskolin (FSK), increases the amplitude and decay time of spontaneous excitatory post-synaptic currents in cultured hippocampal pyramidal neurones (Greengard *et al.* 1991). This effect is mediated by an increase in the opening frequency and mean open time of non-NMDA receptors. Further studies have pinpointed the consequences of GluR1 phosphorylation. Interestingly, phosphorylation of Ser831 by Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) potentiates single channel conductance (Derkach *et al.* 1999), while phosphorylation of Ser845 by PKA increases open channel probability (Banke *et al.* 2000). Furthermore, these different phosphorylation sites acted upon by different kinases are involved in long-term potentiation (LTP) in a way that depends on the activation history of the synapse (Lee *et al.* 2000). GluR1 phosphorylation is reversible, and several protein phosphatases are involved (Tavalin *et al.* 2002) in a complex and highly regulated equilibrium involving PKA and CaMKII (Lee *et al.* 2000). Recently, it was shown that phosphorylation of GluR1 is necessary for plasticity and learning and memory, since mice with knockin mutations in the GluR1 phosphorylation sites show deficits in LTP and long-term depression (LTD) and have memory defects in spatial learning tasks (Lee *et al.* 2003).

GluR4-containing AMPA receptors are expressed in specific regions of the CNS, where they are responsible for signal transmission at high rates. In the rat hippocampus, GluR4 is expressed early in development and it is the first AMPA receptor subunit to target synapses (Zhu *et al.* 2000). The GluR4 subunit is expressed in most subpopulations of

cat and rat retinal amacrine cells (Qin and Pourcho 1999; Grunder *et al.* 2000), and is the major AMPA receptor subunit present in cultures enriched in chick amacrine-like neurones (Carvalho *et al.* 2002). GluR4 is also the main AMPA receptor subunit expressed in auditory cells of the cochlear nucleus, where the fast kinetics of GluR4 is physiologically important in transmitting the signals necessary for sound localization (Raman *et al.* 1994). The spinal cord motor neurones, where rapid neurotransmission has been recorded (Smith *et al.* 1991), are also enriched in GluR4 (Tomiyama *et al.* 1996). It has been shown that PKC and PKA can phosphorylate GluR4 on Ser842, both *in vitro* and *in vivo*. However, *in vivo* PKA activation leads to a significantly higher phosphorylation of Ser842 than PKC activation (Carvalho *et al.* 1999). Furthermore, in hippocampal slices, PKA phosphorylation of GluR4 on Ser842 was shown to be necessary and sufficient for homomeric GluR4 synaptic delivery (Esteban *et al.* 2003).

In this study, we have evaluated the effect of PKA activation on the activity of native GluR4-containing AMPA receptors, on the phosphorylation of GluR4 and on GluR4 surface expression in cultured chick retina cells. We also investigated whether metabotropic receptors coupled to AC can modulate the activity of AMPA receptors, and the phosphorylation and surface expression of GluR4. Our results show that PKA activation or dopamine D₁ receptor activation leads to increased AMPA receptor activity and increased GluR4 Ser842 phosphorylation and GluR4 surface expression.

Experimental procedures

Materials

Indo-1/acetoxymethyl ester (Indo-1/AM) was obtained from Molecular Probes Inc. (Leiden, the Netherlands). Chelerythrine chloride (CHEL), ionomycin and *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide.2HCl (H-89) were purchased from Calbiochem (San Diego, CA, USA). Forskolin (FSK), 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO-20-1724), *N*⁶,2'-*O*-dibutyryl adenosine-3',5'-cyclic monophosphate, sodium salt monohydrate (db-cAMP) and *Rp*-adenosine-3',5'-cyclic monophosphothioate (*Rp*-cAMPs) were obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA, USA). (±)-1-Phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol (SKF38393) and 2*S*,2'*R*,3'*R*-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV) were obtained from Tocris Cookson Inc. (Bristol, UK). Trypsin was from Gibco Life Technologies (Paisley, UK) and fetal calf serum was from Biochrom KG (Berlin, Germany) or from Biowhittaker (Walkersville, MD, USA). Complete Mini protease inhibitor cocktail and microporous polyvinylidene difluoride (PVDF) membranes were purchased from Roche Diagnostics GmbH (Basel, Switzerland). The rabbit polyclonal anti-GluR4 antibody was obtained from Upstate Biotechnology (Buckingham, UK) and the rabbit polyclonal anti-GluR4 phosphorylated at Ser842 antibody was produced against the chemically phosphorylated peptide (RNKARLSPITGGSV) corresponding to rat GluR4 C-terminal 12

amino acids (836–847) phosphorylated at Ser842 (Esteban *et al.* 2003). The alkaline phosphatase-conjugated anti-rabbit and anti-mouse secondary antibodies, and the ECF immunodetection substrate, were obtained from Amersham Biosciences (Uppsala, Sweden). EZ-link Sulfo-NHS-SS-biotin, the UltraLink Plus Immobilized Streptavidin Gel and the bicinchoninic acid (BCA) protein assay reagent kit were purchased from Pierce (Rockford, IL, USA). All the other reagents were obtained from Sigma (St Louis, MO, USA) or from Merck (Darmstadt, Germany). Stock solutions of Indo-1/AM, CHEL, RO-20-1724, ionomycin and FSK were made in dimethyl sulfoxide. All the other chemicals were kept in aqueous stocks.

Embryonic chick retina cell culture

Monolayer cultures of chick retina amacrine-like cells were prepared as previously described (Duarte *et al.* 1992, 1996). Briefly, the retinas from 8-day-old chick embryos (White Leghorn) were dissected and digested with 0.1% trypsin, in Ca^{2+} - and Mg^{2+} -free Hank's balanced salt solution, for 15 min at 37°C. The cells were cultured on poly D-lysine (0.1 mg/mL)-coated glass coverslips or tissue culture dishes in basal medium of Eagle (BME), buffered with 25 mM HEPES and 10 mM NaHCO_3 , pH 7.4, and supplemented with 5% heat-inactivated fetal calf serum, penicillin (100 U/mL) and streptomycin (100 µg/mL). The cells were maintained at 37°C in a humidified incubator with 95% air and 5% CO_2 , and used after 5 days in culture.

Indo-1 loading and Ca^{2+} measurements

$[\text{Ca}^{2+}]_i$ was measured by Indo-1 fluorescence, as described previously (Duarte *et al.* 1996; Carvalho *et al.* 1998), using a computer-assisted Perkin-Elmer LS-5B luminescence spectrometer with fluorescence excitation at 335 nm and emission at 410 nm, and using 5 nm slits. Monolayers of chick retina cells cultured on coverslips, at a density of 0.6×10^6 cells/cm², were incubated with 3 µM Indo-1/AM in BME buffered with 25 mM HEPES, for 45 min at 37°C, and further incubated in BME, for 15 min, in the absence of the dye, to ensure complete hydrolysis of Indo-1/AM. The glass coverslips were then mounted in a stirred, thermostatted (37°C) cuvette, and the fluorescence emission intensities were recorded every 0.5 s. The $[\text{Ca}^{2+}]_i$ was calculated from the emission fluorescence values using the equation for single fluorescence measurements (Grynkiewicz *et al.* 1985; Bandeira-Duarte *et al.* 1990). The calibration was performed at the end of each experiment, using 3 µM ionomycin to obtain maximal fluorescence, followed by addition of 2 mM MnCl_2 to obtain autofluorescence. The dissociation constant taken for the Indo-1/ Ca^{2+} complex was 250 nM (Grynkiewicz *et al.* 1985).

Stimuli

Cells were washed and stimulated as indicated in *N*-methyl-D-glucamine (NMG) medium (132 mM NMG; 4 mM KCl; 1 mM CaCl_2 ; 1.4 mM MgCl_2 ; 6 mM glucose; buffered with 10 mM HEPES; pH 7.4). A Na^+ -free medium was used in order to prevent cell depolarization following AMPA receptor activation, ruling out calcium entry through voltage-dependent calcium channels and therefore isolating the component due to Ca^{2+} influx through activated AMPA receptors. To allow correlation with the $[\text{Ca}^{2+}]_i$ data, NMG medium was also used in the experiments performed to

determine GluR4 phosphorylation on Ser842 and GluR4 surface expression.

In all experiments, including the controls, cells were incubated with a selective inhibitor of cAMP phosphodiesterase, RO-20-1724 (1 µM), throughout the experiment (5 min), to prevent cAMP degradation.

GluR4 phosphorylation

After incubation with the indicated stimuli, cells were washed twice with NMG medium, lysed with ice-cold RIPA buffer [150 mM NaCl, 50 mM Tris-HCl, 5 mM EGTA, 1% Triton X-100, 0.5% deoxycholic acid (DOC), 0.1% sodium dodecyl sulfate (SDS); pH 7.5] supplemented with protease inhibitors (Complete Mini protease inhibitor cocktail) and phosphatase inhibitors (10 mM Na_4PO_7 ; 50 mM NaF; 1 mM Na_3VO_4), and scraped off the culture dishes. The lysates were centrifuged (14 000 g, 10 min, 4°C) and the pellet was discarded. Protein concentration was measured using the BCA protein assay reagent kit (Pierce) and the protein concentration was equalized among samples. The samples were then diluted 4 : 1 with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer [final concentration: 0.125 M Tris, 2% (w/v) SDS, 5% (v/v) glycerol, 5% (v/v) β-mercaptoethanol] and boiled for 10 min.

Biotinylation of plasma membrane-associated proteins

Cells were washed twice with phosphate-buffered saline (PBS) with calcium and magnesium ($\text{PBS}/\text{Ca}^{2+}/\text{Mg}^{2+}$: 137 mM NaCl; 2.7 mM KCl; 1.8 mM KH_2PO_4 ; 10 mM Na_2HPO_4 ; plus 0.5 mM MgCl_2 ; 1 mM CaCl_2 ; pH 7.4), and then incubated with 1 mg/mL NHS-SS-Biotin for 30 min at 4°C under mild shaking. They were then rinsed three times with $\text{PBS}/\text{Ca}^{2+}/\text{Mg}^{2+}$ supplemented with glycine (100 mM) and a fourth time with PBS supplemented with protease inhibitors. Cells were then lysed with RIPA buffer supplemented with protease and phosphatase inhibitors, scraped off the plates and centrifuged at 14 000 g for 10 min at 4°C. The supernatant fluids were transferred to clean tubes and protein concentration was measured using the BCA protein assay reagent kit. Ultra-Link Streptavidin Plus was added to equal amounts of supernatant fluid (2 µg/10 µg total protein) and incubated for 2 h at 4°C with mild shaking (orbital shaker). Complexes were then centrifuged (2500 g, 3 min) and the supernatant fluid, corresponding to intracellular proteins, was collected. Complexes were washed further with RIPA buffer and centrifuged (2500 g, 3 min) four times. Proteins were eluted from streptavidin beads by boiling for 10 min in SDS-PAGE sample buffer.

SDS-PAGE and immunoblotting

The extracts obtained were resolved by SDS-PAGE in 10% polyacrylamide (Laemmli 1970) gels using 30–35 µg protein per well. This was followed by overnight electrotransfer to PVDF membranes, at 40 V, complemented by 30 min at 200 V. Membranes were then blocked for 1 h with 1% (w/v) bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween-20 (TBS-T), and probed for 1 h with the primary anti-GluR4 (1 : 400) or anti-GluR4 phosphorylated at Ser842 (1 : 2500) antibodies. Following five washes (5 min) in 1% BSA/TBS-T, the membranes were incubated for 1 h with alkaline phosphatase-conjugated secondary anti-rabbit antibody (1 : 20 000). The membranes were then washed again five times (5 min), incubated with chemifluorescence substrate

(ECF) for 5 min, and scanned with the Storm 860 scanner (Amersham Biosciences). The scanned digital images were quantified using the ImageQuant 5 software (Amersham Biosciences).

Statistical analysis

Results are presented as means \pm SEM of the indicated number of experiments, carried out in different preparations. Statistical significance was determined by one-way ANOVA followed by the Dunnett's or Bonferroni's test.

Results

Modulation of Ca^{2+} influx through AMPA receptors by PKA

In previous studies, we have shown that kainate increases the $[\text{Ca}^{2+}]_i$ in cultured chick retina cells due to activation of AMPA receptors (Carvalho *et al.* 1998). These cells contain Ca^{2+} -permeable AMPA receptors (Carvalho *et al.* 1998, 2002), and activation of AMPA receptors in a Na^+ -containing medium leads to Ca^{2+} entry through the receptor-associated channels and through voltage-gated Ca^{2+} channels activated by depolarization (Duarte *et al.* 1996). To avoid cell depolarization upon receptor activation, all experiments were performed in a Na^+ -free NMG medium. Under these circumstances, receptor activation leads to Ca^{2+} entry only through AMPA receptor-associated channels (Duarte *et al.* 1996; Carvalho *et al.* 1998). Therefore, the $[\text{Ca}^{2+}]_i$ response to kainate in primary cultures of chick amacrine-like neurones is a good experimental model for studying the regulation of Ca^{2+} -permeable, AMPA-type glutamate receptor activity (Duarte *et al.* 1996; Carvalho *et al.* 1998).

To examine whether the activity of AMPA receptors is regulated by PKA, we measured the effect of FSK and of db-cAMP on the $[\text{Ca}^{2+}]_i$ response to kainate (Fig. 1b). To better unravel the effect of activating PKA, a phosphodiesterase inhibitor, RO-20-1724, was present in the experimental medium throughout the experiment. Representative traces of the effect of AC activation with FSK on the influx of Ca^{2+} through AMPA receptors are shown in Fig. 1(a). Pre-incubation of the cells with 10 μM FSK led to an increase of $24.0 \pm 4.5\%$ on the $[\text{Ca}^{2+}]_i$ rise evoked by kainate relatively to the control response (Fig. 1b). FSK activates AC, leading to an increase of cAMP and subsequent activation of PKA. A similar effect was observed when PKA was activated with db-cAMP (1 mM), a cell permeable cAMP analogue ($35.3 \pm 8.18\%$ above control; Fig. 1b). On the other hand, pre-incubation of amacrine-like neurones with 500 μM Rp-cAMPs, a selective competitive inhibitor of PKA, before activation of AMPA receptors with kainate, caused a decrease of $23.1 \pm 5.6\%$ on the $[\text{Ca}^{2+}]_i$ response to the agonist (Fig. 1b). Thus, endogenous PKA actively modulates AMPA receptors in cultured retinal neurones.

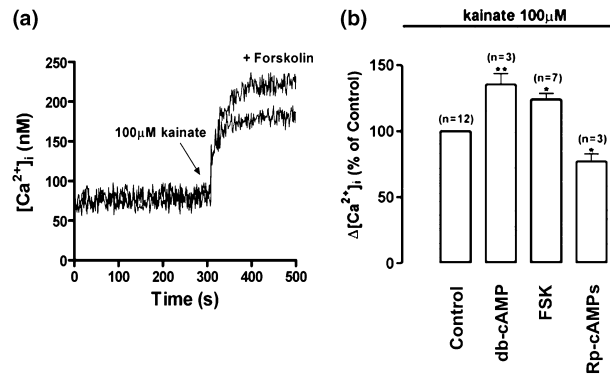


Fig. 1 Effect of PKA activation or inhibition on the $[\text{Ca}^{2+}]_i$ response to kainate in Na^+ -free extracellular medium, in cultured chick amacrine-like neurones. (a) Representative traces of potentiation of the kainate-evoked Ca^{2+} influx by FSK. The cells were incubated with or without 10 μM FSK 3 min before stimulation of AMPA receptors with 100 μM kainate. (b) The cells were pre-incubated with the PKA activator db-cAMP (1 mM) for 1 h, with FSK (10 μM) for 3 min, or with PKA inhibitor Rp-cAMPs (500 μM) for 1 h, before stimulation with 100 μM kainate. Data are expressed as percentage of control of the maximal $[\text{Ca}^{2+}]_i$ response ($\Delta[\text{Ca}^{2+}]_i$) to 100 μM kainate and are means \pm SEM of the indicated number of experiments carried out at least in duplicate in different preparations. * $p < 0.05$, ** $p < 0.01$, Dunnett's test.

Effect of dopamine D_1 receptors and group II mGluR on AMPA receptor activity

Since PKA modulates the kainate-evoked Ca^{2+} influx through AMPA receptors, we looked for physiological stimuli that could act upstream of PKA. For this purpose, we tested whether AMPA receptor activity is changed by activation of dopamine D_1 receptors, which activate AC via G proteins, increasing the intracellular cAMP content and thereby activating PKA, and of group II mGluR that inhibit AC. Both types of metabotropic receptors are expressed in chick retinal neurones (de Mello *et al.* 1996; Caramelo *et al.* 1999).

Activation of dopamine D_1 receptors with the selective agonist SKF38393 (10 μM) (Noh and Gwag 1997) increased the $[\text{Ca}^{2+}]_i$ response to kainate by $20.2 \pm 6.8\%$ compared with the control (Fig. 2a). This increase was totally blocked by pre-incubation of the cells with a D_1 receptor antagonist (SKF83566; 10 μM) (Noh and Gwag 1997) or Rp-cAMPs (a PKA inhibitor) before stimulation with SKF38393 (Fig. 2a). This shows that the potentiation of Ca^{2+} influx through AMPA receptors by SKF38393 was due to dopamine D_1 receptor activation and was mediated via PKA.

Figures 2b and 2c show the effect of group II mGluR activation, with the selective agonist DCG-IV (1 μM) (Brabet *et al.* 1998), on the $[\text{Ca}^{2+}]_i$ response to kainate. DCG-IV did not significantly affect the influx of Ca^{2+} through AMPA receptors ($0.3 \pm 1.4\%$ above control,

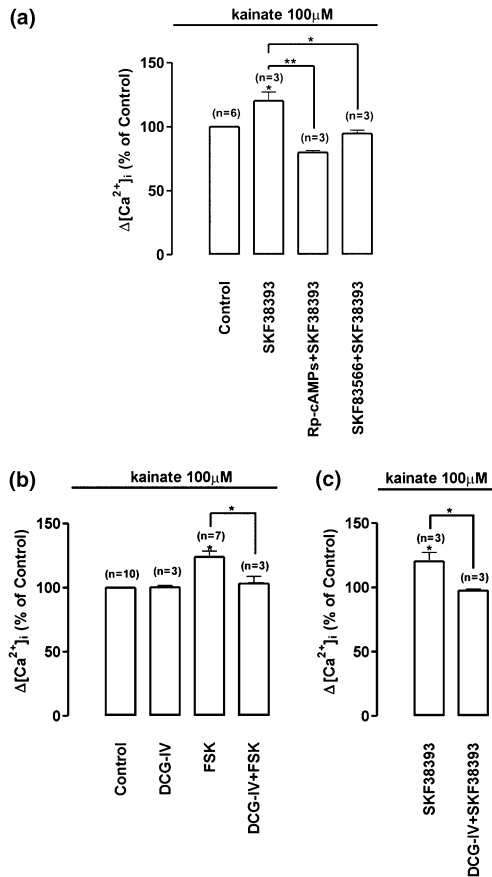


Fig. 2 Effect of dopamine D₁ receptors and group II mGluR activation on Ca²⁺ influx through AMPA-type glutamate receptors in response to kainate, in Na⁺-free medium. (a) Effect of dopamine D₁ receptor activation was measured after pre-incubation with the D₁ receptor specific agonist SKF38393 (10 μM) for 3 min. When Rp-cAMPS (500 μM) was used, the cells were pre-incubated with the kinase inhibitor for 1 h before stimulation with SKF38393. Inhibition of dopamine D₁ receptors was carried out with SKF83566 (10 μM) for 4 min, before addition of the receptor agonist. (b) and (c) Effect of Group II mGluR activation with DCG-IV (1 μM) for 4 min, and effect of pre-incubation with DCG-IV on the response to AC stimulation with FSK (b) or D₁ receptor activation with SKF38393 (c). The cells were incubated with FSK (10 μM) or SKF38393 (10 μM) 3 min before stimulation with kainate. Data are expressed as percentage of control of the maximal $[Ca^{2+}]_i$ response ($\Delta[Ca^{2+}]_i$) to 100 μM kainate, and are means \pm SEM of the indicated number of experiments carried out at least in duplicate in different preparations. * $p < 0.05$, ** $p < 0.01$, Bonferroni's test.

Fig. 2b). However, an interesting effect was seen when DCG-IV was added to the cells before FSK (Fig. 2b) or SKF38393 (Fig. 2c). DCG-IV inhibited the up-regulatory effects of FSK ($3.1 \pm 5.6\%$ above control) and SKF38393 ($-2.5 \pm 1.1\%$) on AMPA receptor-mediated Ca²⁺ influx. These results show that there is a functional cross-talk between mGluR and dopamine receptors leading to modulation of AMPA receptor activity.

Phosphorylation of GluR4 at Ser842

GluR4 is the most abundant AMPA-type glutamate receptor subunit present in cultured amacrine-like neurones (Carvalho *et al.* 2002), and PKA can phosphorylate GluR4 on Ser842 *in vitro* and in transfected cells (Carvalho *et al.* 1999). To examine the effect of PKA activation on the phosphorylation state of native GluR4, we stimulated cultures enriched in retinal amacrine-like cells as indicated previously. The extracts obtained were then submitted to SDS-PAGE, followed by immunoblot with a phosphorylation site-specific antibody against GluR4 phosphorylated on Ser842. This antibody is blocked by the phosphorylated antigenic peptide and does not show cross reactivity with unphosphorylated GluR4 (Esteban *et al.* 2003). Equal amounts of protein were applied to all lanes and the quantification of phosphorylated GluR4 was normalized according to the amount of total GluR4 in each lane.

In control conditions, phosphorylated GluR4 migrated as a band of approximately 110 kDa, showing that there was basal phosphorylation of GluR4 (Fig. 3).

There was a clear increase in GluR4 phosphorylation after exposure of retinal neurones to 10 μM FSK in the presence ($83.17 \pm 7.9\%$ above control; Fig. 3a) or in the absence ($95.6 \pm 3.4\%$ above control; data not shown) of the phosphodiesterase inhibitor RO-20-1724. This potentiation was not significantly affected by pre-incubating the cells with CHEL (5 μM), a specific protein kinase C (PKC) inhibitor (Herbert *et al.* 1990) ($60.7 \pm 13.9\%$ above control; Fig. 3a). In contrast, incubation with 1 μM H-89 (Davies *et al.* 2000), an inhibitor of PKA, decreased GluR4 phosphorylation to $64.5 \pm 9.2\%$ of control (Fig. 3a), indicating that basal PKA activity is important for phosphorylation of the receptors in resting conditions. This decrease in GluR4 phosphorylation after incubation with H-89 was also observed when the cells were stimulated with FSK after pre-incubation with the PKA inhibitor ($69.1 \pm 2.7\%$ of control; Fig. 3a).

The dopamine D₁ receptor agonist (SKF38393) also increased the phosphorylation of GluR4, to $53.5 \pm 3.8\%$ above the control (Fig. 3b). Similar results were observed in experiments where the cells were stimulated with the D₁ receptor agonist in the absence of the phosphodiesterase inhibitor ($62.5 \pm 8.6\%$ above control; data not shown), indicating that this effect was not due to a cell overload with cAMP following inhibition of phosphodiesterases. Moreover, the increase in GluR4 phosphorylation induced by SKF38393 was completely abolished by pre-incubating the cells with 1 μM H-89 ($72.3 \pm 4.2\%$ of control; Fig. 3b). These results indicate that GluR4 phosphorylation at Ser842, induced both by FSK and D₁ receptor activation with SKF38393, occurs via PKA.

Activation of group II mGluR with the selective agonist DCG-IV (1 μM) slightly decreased GluR4 phosphorylation, although this effect was not statistically significant (Fig. 3c). However, DCG-IV inhibited the effect of both FSK and

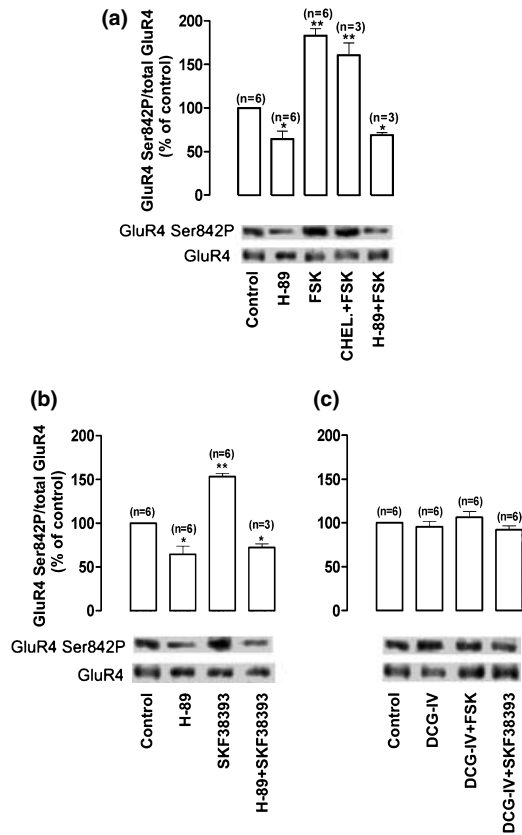


Fig. 3 GluR4 phosphorylation at Ser842. Monolayer cultures of chick embryo retina amacrine-like neurones were subjected to different conditions, as explained, and the phosphorylation of GluR4 at Ser842 was evaluated. (a) Effect of PKA activation and inhibition on GluR4 phosphorylation at Ser842. Cells were exposed to FSK (10 μ M) for 3 min, or to H-89 (1 μ M) for 10 min, prior to preparation of cell extracts. To determine whether the effect of FSK is through activation of PKA, cells were pre-incubated with H-89 for 7 min, before stimulation with FSK. Pre-incubation of the cells with CHEL (5 μ M), a PKC inhibitor, did not affect the effect of FSK on GluR4 Ser842 phosphorylation. (b) To evaluate the role of dopamine D₁ receptor activation on the phosphorylation of GluR4 by PKA, cells were incubated with SKF38393 (10 μ M) for 3 min, or with H-89 (1 μ M) for 7 min, prior to incubation with SKF38393. (c) Effect of group II mGluR on GluR4 phosphorylation. Cells were incubated with DCG-IV (1 μ M) for 4 min before cell lysis. For the pre-activation of group II mGluRs, cells were pre-exposed to DCG-IV (1 μ M) for 1 min, followed by a 3 min exposure to DCG-IV and FSK (10 μ M) or to DCG-IV and SKF38393 (10 μ M). Cell extracts were prepared, submitted to SDS-PAGE and immunoblotted against phosphorylated GluR4 at Ser842 and total GluR4. Total GluR4 and phosphorylated GluR4 on Ser842 were quantified and the amount of phosphorylated GluR4 was normalized to the total amount of GluR4 in each lane. All data are expressed as percentage of control and plotted as the mean \pm SEM, for the indicated number of experiments performed in independent preparations (* p < 0.05, ** p < 0.01, Bonferroni's test). Representative western blots, using antibodies against phosphorylated GluR4 at Ser842 and total GluR4, are shown.

SKF38393. Accordingly, following pre-incubation with DCG-IV, FSK did not change the phosphorylation state of GluR4 significantly (+ 6.5 \pm 6.6%; p > 0.05; Fig. 3c) relative to control conditions. Similar results were obtained when the cells were pre-incubated with DCG-IV before stimulation with SKF38393 (7.6 \pm 4.4% below control; p > 0.05; Fig. 3c).

GluR4 surface expression

Receptor trafficking has been proposed to underlie synaptic plasticity phenomena and, in particular, AMPA receptor subunits have been shown to recycle continuously between intracellular compartments and the plasma membrane (reviewed in Sheng and Lee 2001; Malinow and Malenka 2002; Song and Huganir 2002; Gomes *et al.* 2003). In order to investigate whether GluR4 was mobilized to the membrane as a consequence of PKA activation and GluR4 phosphorylation, we performed a biotinylation assay, in control cells and in retinal neurones exposed to stimuli which regulate PKA activity, directly or through stimulation of metabotropic receptors, and looked for changes in the plasma membrane-associated GluR4 and phosphorylated GluR4 at Ser842.

Stimulation of AC with FSK (10 μ M) resulted in a clear increase both in phosphorylated GluR4 at Ser842 (58.3 \pm 5.5% above control) and total GluR4 (42.5 \pm 7.1% above control) (Fig. 4a) in the biotinylated fraction. This effect was not changed by pre-incubating the cells with 5 μ M CHEL, a PKC inhibitor. GluR4 surface expression in cells incubated with the dopamine D₁ receptor agonist SKF38393 was also investigated. Cell incubation with SKF38393 (10 μ M) resulted in an increase in total GluR4 (31.9 \pm 4.1% above control) and phosphorylated GluR4 (41.1 \pm 5.4% above control) (Fig. 4b) surface expression. However, when cells were incubated with the PKA inhibitor H-89 (1 μ M), or with H-89 prior to FSK or SKF38393, there was a non-significant decrease in the amount of both phosphorylated and total GluR4 (Figs 4a and 4b) at the plasma membrane.

The effect of group II mGluR activation on the trafficking of GluR4 was also investigated by stimulating the amacrine-like neurones with DCG-IV (1 μ M). Although DCG-IV did not affect the levels of plasma membrane-associated GluR4 and phosphorylated GluR4 at Ser842 (Fig. 4c), activation of group II mGluR prevented the effect of FSK and SKF38393.

When supernatant fluids of the biotinylation assay, corresponding to protein in intracellular compartments, were analysed, there were no changes in either phosphorylated or total GluR4 (not shown). This can be explained by the fact that there are substantially more subunits localized intracellularly than on the membrane and therefore, a significant increase in the number of receptors in the membrane will result in only a small relative change in the intracellular population (Hayashi *et al.* 2000).

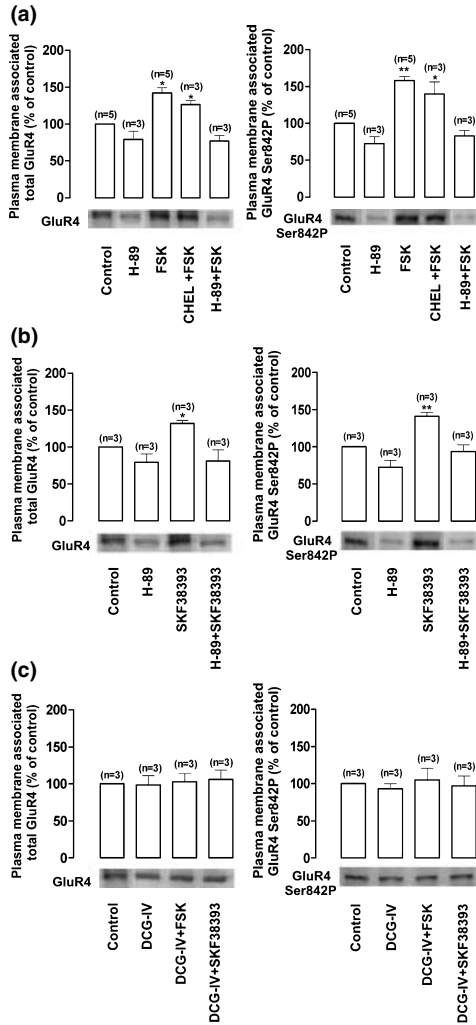


Fig. 4 Effect of PKA activation and metabotropic receptor stimulation on plasma membrane-associated phosphorylated GluR4 at Ser842 and total GluR4. (a) Effect of PKA activation on the surface expression of phosphorylated GluR4 at Ser842 and total GluR4. Primary cultures of chick amacrine-like neurones were incubated with FSK (10 μ M) for 3 min, H-89 (1 μ M) for 10 min, and with CHEL (5 μ M) or H-89 for 7 min, before stimulation of AC with FSK. (b) Role of dopamine D₁ receptor activation on the surface expression of phosphorylated GluR4 at Ser842 and total GluR4. Cells were incubated with SKF38393 (10 μ M) for 3 min, or with H-89 (1 μ M) for 7 min, prior to SKF38393. (c) Effect of group II mGluR on the surface expression of phosphorylated GluR4 and total GluR4. Cells were stimulated with DCG-IV (1 μ M) for 4 min before cell lysis. For the pre-activation of group II mGluRs, cells were pre-exposed to DCG-IV (1 μ M) for 1 min, followed by a 3 min exposure to DCG-IV and FSK (10 μ M) or to DCG-IV and SKF38393 (10 μ M). Biotinylation was performed as described in methods. Streptavidin-retained protein complexes were collected and run on SDS-PAGE. Immunoblot was performed using an antibody against GluR4 phosphorylated at Ser842, and against GluR4 C-terminal. Representative western blots are shown. At least triplicates were run for each condition, using independent preparations, as indicated. Data were normalized and expressed as percentage control. Data are plotted as the mean \pm SEM. ** p < 0.01, * p < 0.05, Bonferroni's test.

Discussion

In the present study, we found that PKA up-regulates Ca²⁺ influx through AMPA-type glutamate receptors in primary cultures of chick retinal neurones. The [Ca²⁺]_i was followed in a Na⁺-free extracellular medium, after activation of AMPA receptors with kainate, a non-desensitizing agonist of AMPA receptors, allowing the measurement of the change in the intracellular calcium concentration in response to AMPA receptor activation. Under these conditions, the rise in [Ca²⁺]_i in response to kainate is due exclusively to Ca²⁺ entry through activated AMPA receptors (Murphy and Miller 1989; Duarte *et al.* 1996; Carvalho *et al.* 1998), since kainate-induced depolarization cannot occur. In fact, in this preparation, the [Ca²⁺]_i response to kainate in NMG-containing extracellular medium was inhibited by 95.4 \pm 2.0% in the presence of 2,3-benzo-diazepine LY 303070, a non-competitive AMPA receptor antagonist (Carvalho *et al.* 1998). Most AMPA receptors in cultured retinal neurones produce a [Ca²⁺]_i response to kainate, since the Ca²⁺-permeable GluR3 and GluR4 subunits are the predominant AMPA receptor subunits in this preparation, and since no GluR2 subunit was detected in these cells (Carvalho *et al.* 2002).

Direct or indirect activation of PKA with db-cAMP or FSK, respectively, increased the kainate-evoked Ca²⁺ influx in a Na⁺-free extracellular medium. In addition, when neurones were incubated with a PKA-specific inhibitor (Rp-cAMPs), there was a decrease in Ca²⁺ influx through AMPA receptors, suggesting that endogenous PKA activity regulates receptor function. This strong modulatory effect of PKA on the influx of Ca²⁺ in kainate-stimulated amacrine-like neurones is consistent with a previous study (Carvalho *et al.* 1998) where it was shown that PKC activity also increases Ca²⁺ influx through AMPA receptors. Accordingly, PKC and PKA can phosphorylate GluR4 on Ser842 *in vitro* and in transfected cells (Carvalho *et al.* 1999). However, PKA can phosphorylate GluR4 at Ser842 to a higher extent than PKC (Carvalho *et al.* 1999), which could explain the stronger modulatory role of PKA in the regulation of AMPA receptor activity in amacrine-like neurones.

Using a phosphorylation site-specific antibody against GluR4 phosphorylated on Ser842, we found a robust increase in GluR4 phosphorylation upon treatment of amacrine neurones in culture with FSK. Phosphorylation of GluR1 is known to increase AMPA receptor response (Greengard *et al.* 1991) by increasing either channel open probability (Banke *et al.* 2000) or single channel conductance (Derkach *et al.* 1999). This direct modulation of channel properties by phosphorylation has not yet been tested for GluR4-containing AMPA receptors, but the FSK-induced increase in GluR4 phosphorylation could be responsible, at least in part, for the up-regulatory effect of PKA on AMPA receptor activity observed by us. Inhibition

of PKA proved equally effective in modulating GluR4 phosphorylation, which suggests a strong PKA dependence of the basal phosphorylation state of GluR4.

Dopamine modulates the activity of non-NMDA ionotropic glutamate receptors in horizontal (Knapp and Dowling 1987) and bipolar retinal cells (Maguire and Werblin 1994). We now show that activation of dopamine D₁ receptors, present in cultured amacrine-like cells (de Mello *et al.* 1996), regulates the activity of AMPA receptors. Dopamine D₁ receptors were found in amacrine cells in both mammalian (Nguyen-Legros *et al.* 1999) and chick (Firth *et al.* 1997) intact retina, which raises the possibility of regulation by D₁ receptors of the activity of AMPA receptors in amacrine cells *in vivo*.

The strong increase in both the [Ca²⁺]_i response to kainate and the phosphorylation of GluR4 following activation of D₁ receptor supports the idea of functional co-localization of dopamine D₁ receptors and AMPA receptors, and should dopamine and glutamate be present in the same synaptic cleft, then the response to glutamate may be affected by dopamine. Dopamine D₁ receptors have also been reported to increase GluR1 phosphorylation on the PKA site Ser845 in cultured neurones from the striatum and the nucleus accumbens, and in neostriatal slices (Price *et al.* 1999; Snyder *et al.* 2000; Chao *et al.* 2002a,b).

The most direct modulation of AMPA receptor activity and GluR4 phosphorylation, however, would be expected to come from metabotropic receptors responding to glutamate itself. As group II mGluR are present in cultured amacrine-like cells (Caramelo *et al.* 1999), we tested the ability of the selective group II mGluR agonist DCG-IV to modulate both entry of Ca²⁺ through activated AMPA receptors and the phosphorylation of the main AMPA receptor subunit in these cells. Group II mGluRs inhibit AC, leading to a decrease of cAMP over time (Pin and Duvoisin 1995), but our results show that DCG-IV did not significantly change the kainate-induced [Ca²⁺]_i response. A major change in the cAMP levels was not expected within the time range of the experiment due to (i) the presence of RO-20-1724, a phosphodiesterase inhibitor, throughout the experiment, preventing degradation of cAMP, and (ii) the slow rate of cAMP formation under resting conditions. Therefore, upstream inhibition of AC is expected neither to affect the pool of cAMP nor modulate the activation state of PKA under the experimental conditions used. Our results show no significant difference in GluR4 phosphorylation in cells incubated with DCG-IV, in agreement with the results concerning the effect on receptor activity. In contrast, inhibition of PKA with H-89 decreased GluR4 phosphorylation, indicating that a certain level of kinase activity is maintained under resting conditions.

However, DCG-IV inhibited to some extent the potentiatory effect of FSK and SKF38393, both in the [Ca²⁺]_i response to kainate (Fig. 2c) and on GluR4 phosphorylation (Fig. 3c). The reduction of GluR4 phosphorylation and the inhibition of AMPA receptor activity, when the cells were

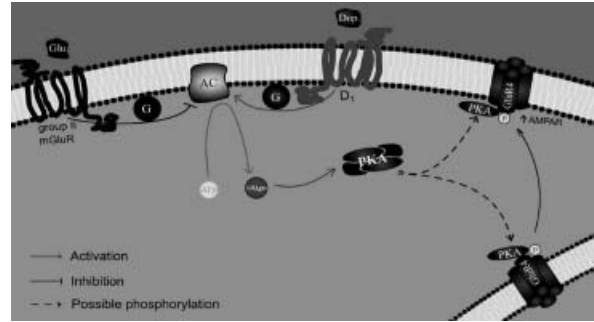


Fig. 5 Dopamine D₁ receptor and group II mGluR regulation of AMPA receptor activity and trafficking via PKA.

incubated with DCG-IV before stimulation of D₁ receptors, is highly significant and provides further evidence for functional co-localization of AMPA receptors, group II mGluRs and D₁ receptors. Moreover, these data show that AC inhibition by group II mGluR is a valid pathway for negative modulation of GluR4 phosphorylation by PKA in the context of receptor cross-talk (Fig. 5). To our knowledge, this is the first time that a cross-talk between dopamine receptors, metabotropic glutamate receptors and AMPA-type ionotropic glutamate receptors has been reported.

AMPA receptor trafficking is known to play a role in synaptic plasticity and in unsilencing of synapses (reviewed in Barry and Ziff 2002; Malinow and Malenka 2002; Song and Huganir 2002; Gomes *et al.* 2003). Our results show that there was a clear increase in GluR4 at the plasma membrane (Fig. 4) when cells were exposed to stimuli that led to an increase in GluR4 phosphorylation (FSK and SKF38393). Also, a decrease in surface expression was observed when GluR4 phosphorylation was diminished, showing a very good correlation between the phosphorylation of GluR4 at Ser842 by PKA and surface expression of this AMPA receptor subunit. An increase in the number of receptors in the membrane following PKA activation may suggest that there is modulation of receptor turnover by increasing the rate of receptor delivery to the plasma membrane and/or by decreasing the rate of receptor removal. However, the effect of PKA activation on GluR4 expression at the plasma membrane did not fully correlate with the increase in AMPA receptor activity. This apparent discrepancy may be due to the fact that in [Ca²⁺]_i measurements, the signal comes mainly from the cell body, which represents a large percentage of the cell volume, whereas the measurements of GluR4 at the plasma membrane take into consideration the population of receptors present in the cell body and those at the processes.

In the hippocampus, GluR1 and GluR4, the subunits containing long cytoplasmic tails, seem to be restricted from accessing synapses until activation by LTP or spontaneous activity, respectively, release this restriction, allowing GluR1- or GluR4-containing receptors to be inserted into synapses. GluR4 is expressed in the hippocampus earlier in

development than any other subunit, and its expression is restricted to the first post-natal week, decreasing steadily as the other subunits, GluR1-3, rise until only the latter are expressed significantly (Zhu *et al.* 2000). Spontaneous neuronal activity selectively delivers GluR4-containing AMPA receptors to synapses. Subsequently, these receptors are exchanged by GluR2/GluR3-containing receptors. Therefore, GluR4 delivery to synapses, triggered by spontaneous activity, seems to be crucial for the initial establishment of functional synapses. A recent report shows that PKA activation in hippocampal organotypic slices transiently expressing GluR4 is both necessary and sufficient for the delivery of GluR4-containing receptors into synapses (Esteban *et al.* 2003), and suggests that GluR4-containing receptors are excluded from synapses by means of a retention interaction that is disrupted upon PKA phosphorylation. Our data showing a PKA-induced increase in plasma membrane-associated phosphorylated and total GluR4 (Fig. 4), that is naturally expressed in amacrine-like neurones, are consistent with the hippocampal model and suggest that the same retention system can be at work here. Our results further suggest that the retention interaction that is removed by PKA phosphorylation of GluR4 acts by preventing surface expression of GluR4-containing receptors, rather than by keeping the receptor at an extrasynaptic location at the plasma membrane. Therefore, PKA phosphorylation of GluR4 leads to GluR4 insertion at the plasma membrane, rather than to receptor recruitment from extrasynaptic to synaptic sites.

We report a cross-talk between two types of metabotropic receptors (dopamine D₁ receptors and group II mGluR) and AMPA receptors leading to the modulation of AMPA receptor activity. We also show that upon stimulation of PKA, directly or through dopamine D₁ receptor activation, and GluR4 phosphorylation at Ser842, there is an increase in total and phosphorylated GluR4 in the plasma membrane. However, phosphorylation of GluR4 present in AMPA receptors at the plasma membrane may change the channel characteristics of the receptor, analogously to what was shown for GluR1 (Derkach *et al.* 1999; Banke *et al.* 2000). It remains to be determined to what extent the phosphorylation *per se* of plasma membrane-associated AMPA receptors, and the trafficking of receptors to the membrane, contribute to the observed potentiation of AMPA receptors upon PKA activation.

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