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Int. J. Devl Neuroscience 25 (2007) 499–508

INTERNATIONAL JOURNAL of DEVELOPMENTAL NEUROSCIENCE

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Signal transduction pathways associated with ATP-induced proliferation of cell progenitors in the intact embryonic retina

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Received 26 June 2007; received in revised form 29 August 2007; accepted 18 September 2007

Abstract

ATP and ADP induce retinal cell proliferation through activation of PKC and extracellular signal-regulated kinases (ERKs). Here, we characterized the effect of purinergic agonists on the turnover of phosphoinositides and activation of ERKs during development of the chick embryo retina. When intact retinas were incubated with ATP, ADP or UTP, a dose-dependent accumulation of [^{3}H]-phosphoinositides was observed (% of control, EC_{50}: 548 ± 20.5%, 0.18 mM; 314 ± 53.8%, 0.51 mM; 704 ± 139.9%, 0.018 mM, respectively). Only the response promoted by ADP was completely inhibited by the P2 receptor antagonists, PPADS and suramin. All the responses decreased with the progression of retinal development. Western blot assays revealed that ATP, ADP and UTP stimulated the phosphorylation of ERKs in the chick embryo retina very early during development (% of control: 174 ± 16; 199 ± 16.4 and 206 ± 37, respectively). The responses to ADP and UTP were transient and dose-dependent, showing EC_{50} values of 0.12 mM and 0.009 mM. The response to ADP was inhibited by the antagonists PPADS and suramin and by U73122 and chelerythrine chloride, which block PLC and PKC, respectively. Conversely, chelerythrine chloride did not block the response induced by UTP. Immunohistochemical analysis revealed that ATP and ADP induced the phosphorylation of ERKs in cells of the neuroblastic layer of retinas from embryos at E8. Our data showed that ATP, ADP and UTP stimulate the turnover of InsPs and promoted the activation of ERKs in the chick embryo retina. ADP, through activation of $P2Y_1$ receptors, activated ERK pathway through PLC and PKC and UTP, via $P2Y_4$-like receptors, induced the phosphorylation of ERKs through a pathway that did not involve PKC.

Keywords: P2 receptors; Nucleotides; Proliferation; ERK; Phosphoinositides; Chick retina

1. Introduction

In the central nervous system (CNS), including the retina, nucleotides such as ATP and UTP act as extracellular signaling molecules by activation of membrane bound P2 receptors (for review, Burnstock and Knight, 2004; Von Kügelgen and Wetter, 2000). Two families of purinergic receptors sensitive to ATP and its analogs have been identified: ligand-gated ion channels that constitute the P2X family and G protein-coupled receptors that form the P2Y family (Ralevic and Burnstock, 1998).

Within early stages of development of the neural chick retina, ATP and UTP act on progenitor cells to evoke Ca^{2+} transients (Sugioka et al., 1996; Sakaki et al., 1996; Catsicas et al., 1998; Yamashita and Sugioka, 1998; Pearson et al., 2002; Webb and Miller, 2003) and to speed their mitosis (Sugioka et al., 1999b; Pearson et al., 2002), an effect that leads to enhanced proliferation and bigger eyes. Recently, Pearson et al. (2005) have shown that the retinal pigment epithelium (RPE) releases ATP by efflux through gap junction connexin 43 hemichannels speeding cell division in the neural retina between stages E3 and E7. This effect is mimicked by UTP and blocked by suramin and PPADS, suggesting a crucial role for P2Y2 receptors in the generation of retinal neurons. These cells are likely to be ganglion, amacrine, photoreceptors and horizontal cells, since the generation of bipolar and glial Müller cells occurs later (Prada et al., 1991).

ATP may also be involved in the induction of proliferation of retinal progenitors other than those affected by UTP. It has been previously demonstrated that ATP and ADP, but not UTP,

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0736-5748/$30.00
doi:10.1016/j.ijdevneu.2007.09.007

induced cell proliferation in monolayer cultures of developing cells obtained from retinas of 6- to 9-day-old chick embryos. This effect was blocked by suramin and PPADS, suggesting that the P2Y1 receptor mediated this proliferative effect of ATP in the monolayer cultures (Sanches et al., 2002). However, ATP-induced proliferation of retinal cells in organ cultures was only demonstrated in tissues from early developing 3- or 4-day-old embryos (Sugioka et al., 1999b), but not in tissues from older embryos at E7 or E8.

The ERK pathway is a membrane-to-nucleus signaling module involved in multiple physiological processes, including cell proliferation, cell differentiation and apoptosis (Schaeffer and Weber, 1999; Peyssonnaux and Eychène, 2001). The effect of ATP on the proliferation of retinal cells in culture is blocked by U73122, chelerytrine chloride and PD98059, suggesting that the PLC, PKC and ERK pathways mediates the phenomenon (Sanches et al., 2002).

In the present work, we compared the effect of ATP, ADP and UTP on DNA synthesis in chick retinal explants obtained from 8-day-old embryos. We also investigated the intracellular mechanisms involved in the proliferative response of the intact retina to ATP and ADP. We show that, as in dissociated monolayer cultures, ATP and ADP, but not UTP, increase the incorporation of [^{3}H]-thymidine in chick retinal explants. We also show that ATP, ADP and UTP induce the accumulation of [^{3}H]-phosphoinositides and that ADP and UTP induce a transient activation of the ERK pathway. Phospho-ERK and BrDU labeled cells were detected at the neuroblastic layer after treatment with ATP and ADP, suggesting that P2Y1 receptors and the ERK pathway mediate the effect of these nucleotides on the proliferation of a population of progenitor cells, which do not proliferate in response to UTP, in the intact chick retina at E8.

2. Experimental procedures

2.1. Materials

[^{3}H]-Thymidine (40 Ci/mmol) and [^{3}H]-*myo*-inositol (22.2 Ci/mmol) were from Amersham Biosciences; ATP, ADP, pyridoxal-phosphate-6-azophenyl-2,4-disulfonic acid (PPADS), UTP and U73122, were from Sigma–Aldrich (St. Louis, MO, USA); PD98059, chelerythrine chloride were from Biomol International (Plymouth Meeting, PA,USA); Basal Medium of Eagle (BME) and Dulbecco's Modified Eagle's Medium (DMEM) were from Invitrogen (Calsbad, CA, USA). Monoclonal anti-phospho-ERK antibody was from Cell Signaling Tech. (MA, USA) and polyclonal anti-ERK antibody was from Promega Inc. (Madison, WI, USA). All other reagents were of analytical grade.

2.2. Accumulation of [^{3}H]-phophoinositides ([^{3}H]-InsPs)

Retinas from embryos at different stages of development were incubated for 2.5 h, at 37 °C, in 1 ml of inositol-free DMEM, containing 1 μCi [^{3}H]-*myo*-inositol. LiCl, to a final concentration of 10 mM, was added 1.5 h after beginning of incubation. After the end of a 2.5 h incubation, nucleotides were added and the tissues further incubated for an additional 1 h period. Incubations were interrupted by washing the retinas three times with 2 ml of Hank's balanced salt solution and by subsequent addition of trichloroacetic acid (TCA, 15% final concentration). Protein content of the samples was removed by centrifugation at 27,000 × *g* for 30 min. The pellet was dissolved in 1N NaOH and quantified as described (Lowry et al., 1951). The aqueous phase of the samples was washed 3 times with ether and neutralized with an equal volume of 20 mM Tris–HCl (pH 7.4), containing 5 mM EDTA. The mixture was added to a 1 ml Dowex X-8 column (formate form). Fractions were sequentially eluted with water, 5 mM sodium tetraborate/60 mM ammonium formate and 0.1 mM formic acid/1M ammonium formate. This sequence removed [^{3}H]-inositol, [^{3}H]-glycerophosphoinositol and [^{3}H]-InsPs, that were extracted in the same fraction by the "batch" method (Berridge et al., 1983). Radioactivity was determined by liquid scintillation spectroscopy.

2.3. Tissue stimulation and Western blot

Retinas from embryos at E7 and E8 were dissected from other structures of the eye using Ca^{2+}- and Mg^{2+}-free balanced salt solution (CMF) and immediately transferred to culture dishes containing 1 mL of basal medium of Eagle (BME) buffered with 20 mM HEPES, pH 7.4. The dishes were kept on ice until the treatment with agonists. Inhibitors and antagonists were added 15 min prior to stimulation. After addition of nucleotides, retinas were incubated at 37 °C for the appropriate periods and immediately transferred to sample buffer without bromophenol blue. Samples were boiled and centrifuged at 27,000 × *g* for 10 min to remove non-soluble material. Protein content was estimated by the Bradford protein assay (Bradford, 1976), using a BSA solution containing sample buffer as standard. Extract samples (60 μg/lane) were size fractionated on 9% SDS polyacrylamide gels, transferred to PVDF membranes (Amersham Biosciences), stained with Ponceau red and blocked with 5% non-fat milk in Tris buffered saline (pH 7.6) with 0.1% Tween-20. Membranes were incubated with diluted primary antibody (1:2000) for 60 min. Blots were developed using a rabbit anti-mouse IgG serum conjugated with horseradish peroxidase (Sigma Chem. Co.) and enhanced chemiluminescence, according to the manufacturer's protocol (ECL plus, Amersham Biosciences).

2.4. Retinal explants

Explants of chick embryo retinas were prepared as described previously for the rat retina (Rehen et al., 2002). Briefly, chick embryos at E7 were killed instantaneously by decapitation; the retinas dissected in sterile CMF and immediately transferred to culture medium. Fragments of 1 mm^2 size were cut and placed in 5-mL tight-lidded Erlenmeyers containing 3 mL of BME with 2% fetal calf serum. CO_2 was adjusted to 5%. The flasks were kept in an orbital shaker at 80–90 rpm, at 37 °C, for the periods indicated. Treatments were added at the beginning of the incubation period and every time medium was changed.

2.5. [^{3}H]-Thymidine incorporation

Treated explants were incubated with [3H]-thymidine (0.5 μCi) for 60 min, at 37 °C. Explants were then washed two times with 2 mL BME buffered with 25 mM HEPES, pH 7,4 and the tissues incubated overnight, at 4 °C, with 0.4N NaOH and then homogenized. Aliquots of the homogenates were precipitated with 10% trichloroacetic acid (TCA) and collected on Whatmann GF/B glass fiber filters that were washed three times with 5 mL of 5% TCA. Filters were dried and the radioactivity determined by scintillation spectroscopy. Protein was estimated by the method of Lowry et al. (1951), using bovine serum albumin as standard.

2.6. Labeling of cycling cells with BrdU

To distinguish between proliferating and postmitotic cells we used 5-bromo-2′-deoxyuridine (BrdU, Sigma, St. Louis, MO, USA), that is incorporated instead of thymidine by cells synthesizing DNA (Gratzner, 1982). Retinal explants were incubated with 3 μg/mL BrdU in the last 1 h of culture, at 37 °C. Prior to immunochemistry with anti-BrdU antibody, the explants were fixed by immersion with 4% paraformaldehyde in sodium phosphate buffer pH 7.4 (PBS) for 1 h. After extensive PBS washes, the explants were oriented to allow transverse cut under a dissecting microscope in an aluminum chamber filled with OCT embedding medium. The in situ frozen retina was cut (10 μm) and mounted in slides pre-treated with poly-lysine (200 μg/mL). The slides were heated in citrate buffer at pH 6.0 in a microwave oven for better antigen retrieval (Dover and Patel, 1994) and incubated with 1% hydrogen peroxide for 10 min to inhibit endogenous peroxidase background. Then, slides were incubated with

0.5% Triton X-100 in PBS for 15 min and washed with PBS followed by incubation with a blocking albumin solution (BSA 1% in PBS) for 30 min. Incubation with the monoclonal primary anti-BrdU antibody (RPN 202, Amersham, Little Chalfont, UK) was carried out for 1 h, at room temperature. The sections were washed in PBS, incubated with secondary antibodies and developed with an anti-mouse ABC kit (Vector, Burlingame, CA, USA) using diaminobenzidine (DAB) as chromogen.

2.7. Counting of $BrdU^+$ cells

Dividing cells were recognized at 1000× magnification by their HRP labeling after BrdU staining, in bright field microscopy. The number of $BrdU^+$ cells (mm^2) was reported as the percentage of nuclei that were $BrdU^+$ in the neuroblastic layer and that were counted in fields of 0.14 mm using a Zeiss Axiophot microscope. Counts were made in 3 fields from each of three explants in each group.

2.8. Intact retina fixation and sectioning

Immediately after stimulation, retinas were fixed by immersion in a cold solution of 4% paraformaldehyde (PA) in 0.16 M phosphate buffer (PB), pH 7.2. After 60 min of fixation, the tissues were rinsed with PB and cryoprotected by successive immersion in PBS containing 15% and 30% sucrose. Sections perpendicular to the vitreal surface (20 μm) from control or treated tissues were collected on the same slide.

2.9. Phospho-ERK immunohistochemistry

Slides were washed in PBS and pre-incubated with 5% goat normal serum for 30 min, followed by overnight incubation with monoclonal anti-phospho-ERK antibody (1:400). After several rinses in PBS, sections were incubated, for 2 h, with biotinylated goat anti-mouse IgG (Vector Labs. Inc.) and stained with an HRP-ABC kit (Vector, Burlingame, CA, USA) using DAB as chromogen. Control sections were incubated in the absence of the primary antibody and no immunoreactivity was detected under this condition. Slides always containing section from control and treated tissues were examined and photographed under differential interference contrast illumination in a Zeiss Axioskop microscope. At least three different animals were analyzed for each treatment.

3. Results

Sanches et al. (2002) have demonstrated that the nucleotides ATP and ADP induced an increase in [^{3}H]-thymidine incorporation in retinal cells maintained in vitro as dissociated monolayer cultures. In order to investigate if nucleotides could induce the proliferation of cells in the intact, non-dissociated tissue, the effect of ATP on retinal cell proliferation was characterized in chick retinal explants obtained from 7-day-old embryos (E7). Retinal explants were incubated with 0.1 mM ATP for increasing periods of time (Fig. 1A). After the time points indicated, tissues were processed for [^{3}H]-thymidine incorporation as described in Section 2. An increase in [^{3}H]-thymidine incorporation could already be detected after 6 h of incubation with ATP. ATP-induced incorporation of [^{3}H]-thymidine increased in the next hours of incubation, attaining a maximal incorporation of ∼300% by 24 h of incubation. ATP-induced proliferation decreased thereafter and only a 33% increase in [^{3}H]-thymidine incorporation could be detected by 96 h of explant incubation with ATP.

The effect of ATP decreased after 24 h of incubation of the explants. In order to verify if the decrease in ATP-induced [^{3}H]-thymidine incorporation was due to the degradation of

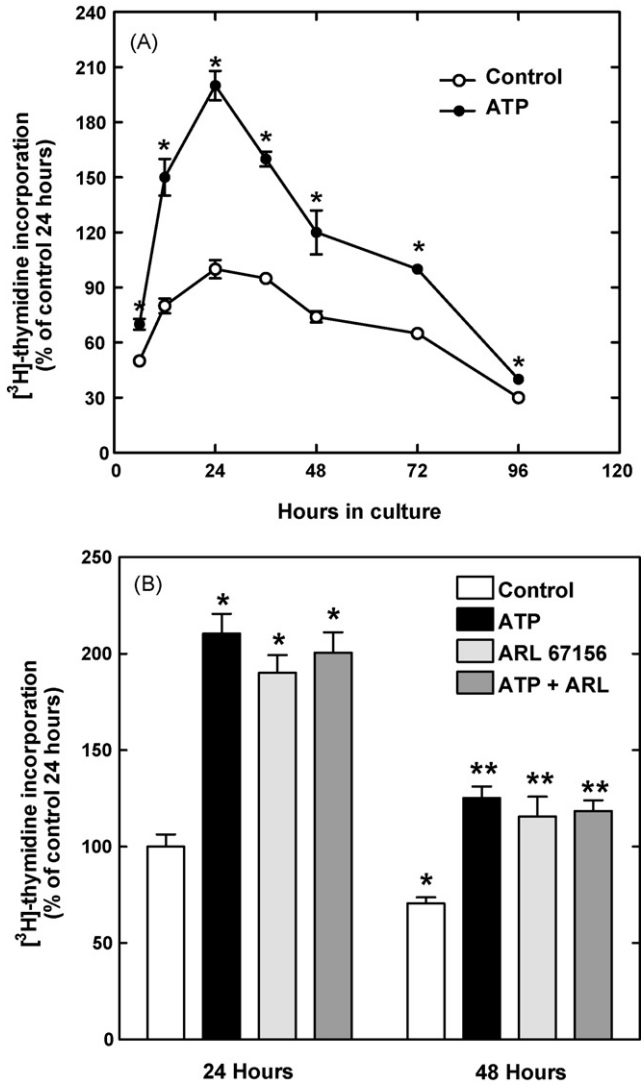

Fig. 1. [^{3}H]-Thymidine incorporation induced by ATP in chick embryo retinal explants. (A) Time course of [^{3}H]-thymidine incorporation induced by ATP. Retinas from 7-day-old chick embryos were dissected, cut in ∼1 mm^2 pieces and cultured as described in Section 2. ATP, at a final concentration of 0.1 mM, was added just after the beginning of the cultures. At the time points indicated, 0.5 μCi of [^{3}H]-thymine was added, the explants incubated for 60 min, washed and processed for analysis of the incorporated radioactivity. Data represent the mean ± S.E.M. of 3 experiments performed in triplicate. $*p < 0.001$, as compared to control. (B) Effect of the ectonucleotidase inhibitor ARL 67156 on ATP-dependent increase in [^{3}H]-thymidine incorporation. Retinal explants from embryos at E7 were cultured for 24 h or 48 h in the presence or absence of 0.1 mM ATP and/or 0.1 mM ARL 67156. After these periods, explants were processed for [^{3}H]-thymidine incorporation. Data represent the mean ± S.E.M. of 3 experiments performed in duplicate. $*p < 0.01$ and $**p < 0.01$ as compared to controls of 24 h and 48 h, respectively.

exogenous nucleotide by ectonucleotidases released from cells, the effect the ectonucleotidase inhibitor ARL 67156 was investigated. Retinal explants were incubated with 0.1 mM ATP in the presence or absence of 0.1 mM ARL 67156 for 24 h or 48 h (Fig. 1B). No attenuation of the ATP-induced increase in [^{3}H]-thymidine incorporation was observed when explants were incubated with the inhibitor for both periods of time. Relative to their respective controls, while ATP induced 210.0% and 177.5% increases after incubations of 24 h and

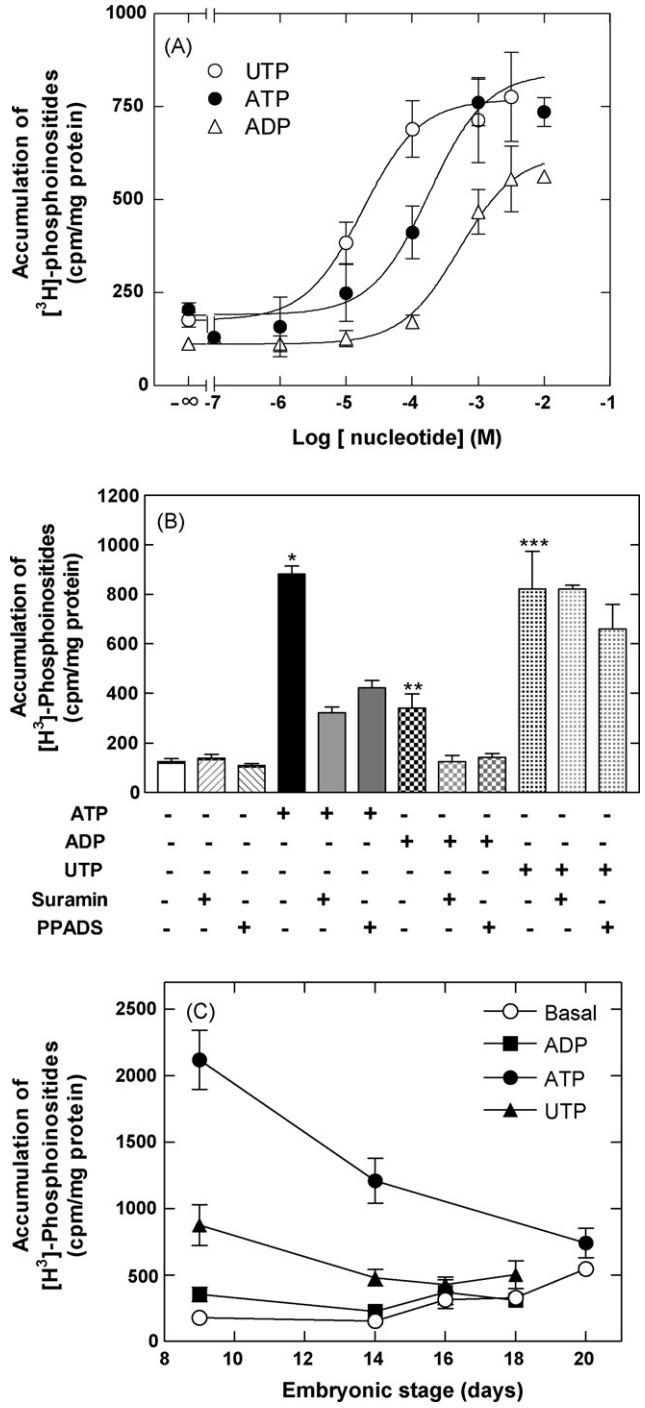

Fig. 2. Accumulation of [^{3}H]-phosphoinositides induced by nucleotides in the chick embryo retina. Tissues from 8-(ADP and UTP) or 9-(ATP) day-old embryos were incubated with 1 μCi of *myo*-[^{3}H]-inositol and 10 mM LiCl for 2.5 h, at 37 °C. The retinas were then stimulated with agonists for an additional 1 h period. Antagonists were added 15 min before stimulation with the nucleotides. Radioactivity was purified and measured as described in Section 2. (A) Retinas were exposed to increasing concentrations of ATP (closed circles), ADP (open triangles) or UTP (open circles). (B) Effect of the P2 antagonists Suramin (Sur) and PPADS on the accumulation of [3H]-phosphoinositides induced by ATP, UTP and ADP. (C) Nucleotide-dependent increase in the accumulation of [^{3}H]-phosphoinositides as a function of the developmental stage of the retina. Retinas from embryos at the indicated embryonic stages were treated with ATP 0.5 mM, ADP 0.5 mM or UTP 0.1 mM. *$p < 0.001$ when compared to basal levels or to ATP + antagonists (Suramin or PPADS); **$p < 0.05$, when compared to basal levels or to ADP + antagonists (Suramin or PPADS); ***$p < 0.001$ when compared to basal levels.

48 h, the increase in [^{3}H]-thymidine incorporation was 200.5% and 167.9% in explants incubated for 24 h and 48 h with ATP plus ARL. Interestingly, explants incubated only with ARL 67156 also showed increases in [^{3}H]-thymidine incorporation similar to the increases observed with ATP (190.1% for 24 h and 163.9% for 48 h).

Previous studies have shown that ATP, acting at the P2Y1 metabotropic receptor, activates the Gq-mediated IP3 signaling pathway, which leads to increases in the levels of intracellular calcium, an event required for cell proliferation (Berridge, 1995; Weissman et al., 2004). In chick embryonic retinal cells in culture, it was also shown that cell proliferation induced by ATP occurs through activation of Gq-coupled P2Y1 receptors (Sanches et al., 2002). To further characterize the presence of Gq-coupled P2 receptors in the embryonic chick retina, we performed the experiments shown in Fig. 2. Intact retinas obtained from chick embryos at E8 or E9 were incubated for 60 min at 37 °C with increasing concentrations of ATP, ADP or UTP. Fig. 2A shows that the 3 nucleotides induced a dose-dependent increase in the accumulation of [^{3}H]-InsPs. Maximal levels of stimulation as % of control were 548 ± 20.5%, 314 ± 53.85% and 704 ± 139.9%, respectively. EC_{50} values were 0.18 mM for ATP, 0.51 mM, for ADP and 0.018 mM for UTP. Retinas were also submitted to the treatment with ATP in the presence of the P2 antagonists Suramin and PPADS (Fig. 2B). While the accumulation of [^{3}H]-InsPs induced by ADP was completely blocked by both antagonists ($p < 0.05$), the effect of ATP was only partially prevented. Moreover, no inhibition of the UTP response was observed with these antagonists.

The ontogeny of the accumulation of [^{3}H]-InsPs induced by nucleotides in the chick embryo retina is shown in Fig. 2C. While basal levels of [^{3}H]-InsPs were low in retinas of embryos from E9 to E14, they increased ~3-fold with the differentiation of the tissue (from 178.1 ± 12.9 cpm/mg protein at E9 to 546.0 ± 47.8 cpm/mg protein at E20). In contrast to basal levels, ATP-induced increase [^{3}H]-InsPs accumulation was highest in tissues from embryos at early embryonic stages and decreased as tissue differentiated, reaching near basal levels by E20 (2118.5 ± 222.7 cpm/mg protein at E9 to 742.2 ± 110.1 cpm/mg protein at E20). The responses to UTP or ADP were also higher in tissues from embryos at E8–E9 and by E14, no significant difference was observed between stimulated and basal levels of [^{3}H]-InsPs.

The ERK pathway is a key element in the signal transduction pathways involved in cellular proliferation and differentiation (Kolch, 2000; Volmat and Pouysségur, 2001). Since PD98059, an inhibitor of MEK1, blocked the mitogenic effect of ATP on retinal cells in cultures (Sanches et al., 2002), we investigated the activation of ERK pathway by nucleotides in the intact chick retina (Figs. 3 and 4). Both ADP and UTP were used as agonists and retinas from embryos at E7 or E8 were submitted to the protocol described in Section 2. Fig. 3A shows the time

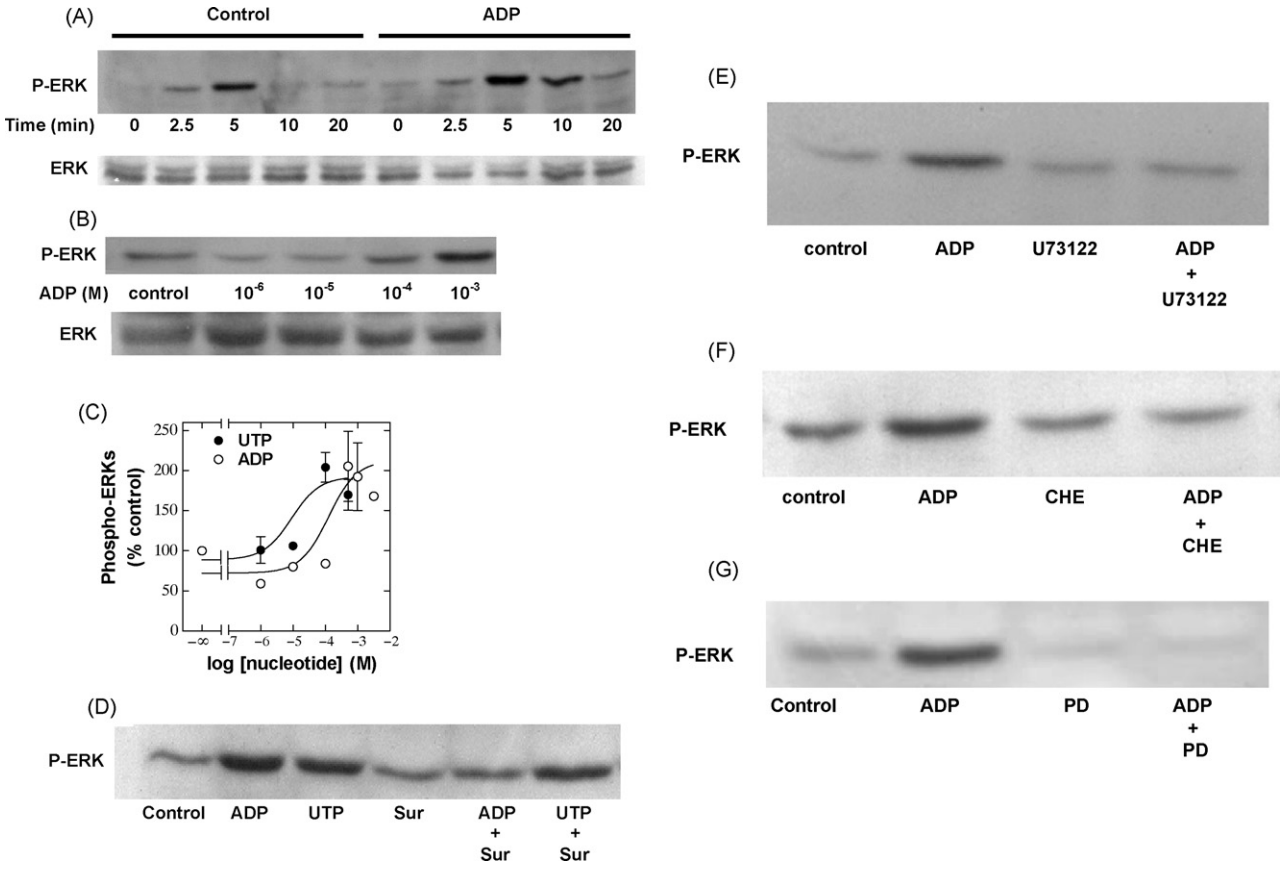

Fig. 3. Activation of the ERK pathway induced by ADP in chick retinas of embryos at E7 or E8. (A) Time-course of ADP-stimulated phosphorylation of ERK (ADP 0.5 mM). Note that both basal and stimulated phosphorylation of ERK peaked at a 5 min incubation. (B) Phosphorylation of ERK as a function of the concentration of the agonist. Retinas were incubated with increasing concentration of ADP for 5 min. (C) Intensity of ERK phosphorylation (% of control) as a function of increasing concentrations of ADP or UTP. Data represent the mean ± S.E.M. of three experiments. (D) Effect of the P2 antagonist suramin (Sur) on ADP or UTP induced-activation of the ERK pathway. (E–G) Effect of U73122 (U), Chelerythrine chloride (CHE), and PD98059 (PD) on ADP-induced activation of the ERK pathway.

course of the phosphorylation of ERK induced by 0.5 mM ADP. Note that retinas showed a transient activation of the ERK pathway, with a peak of activation occuring between 5 and 10 min of stimulation with the nucleotide (in 5 min, levels reached 199 ± 16.4% of the basal; $p < 0.01$, $n = 6$). Basal levels also increased in a time-dependent manner, with a peak of activation also occurring by 5 min of incubation. The response of the retinas to ADP was dose-dependent (Fig. 3B), showing an EC_{50} value for this nucleotide of 0.125 mM (Fig. 3C) and blocked by 0.1 mM suramin, a P2 receptor antagonist (Fig. 3D). The activation of the ERK pathway induced by ADP was also inhibited by 5 μM U73122, 5 μM Chelerythrine chloride and 20 μM PD98059, inhibitors of PLC, PKC and MEK1 enzymes, respectively (Fig. 3E–G).

UTP also induced the activation of the ERK pathway (Fig. 4A). As observed with ADP, retinas showed a transient activation of the ERK pathway, with a peak of activation in 5 min (in 5 min, levels reached 206 ± 37% of basal, $p < 0.001$, $n = 10$). The response was dose-dependent (Fig. 4B) with an EC_{50} of 9.5 μM (Fig. 3C). However, in contrast to what was observed with ADP, the P2 receptor antagonists suramin and PPADS (Figs. 3D and 4C) and the PKC inhibitor chelerythrine chloride did not block the increase in the levels of phospho-ERK induced by UTP (Fig. 4D). As expected, the MEK1 inhibitor, PD98059, completely blocked the effect of UTP (Fig. 4E).

Within the early stages of the development of the neural retina, mitosis is confined to progenitor cells located at the ventricular zone (VZ) of the neuroblastic layer immediately adjacent to the retinal pigment epithelium (RPE) (Pearson et al., 2005). In retinas of E8 chick embryos, the neuroblastic layer lies between the prospective photoreceptor layer and the amacrine cell bodies already located in the inner portion of the prospective Inner Nuclear Layer. To determine whether ERK activation induced by nucleotides was taking place in cells located at the neuroblastic layer in tissues from embryos at E8, imunohistochemistry to phospho-ERK was performed (Fig. 5). In all conditions investigated, including non stimulated retinas, higher immunoreactivity to phospho-ERK was detected over the prospective Inner Plexiform Layer. Only a faint labeling over the neuroblastic layer was detected in control retinal slices maintained in BME for 5 min (Fig. 5A). Treatment of retinas with nucleotides, mainly ADP and ATP, resulted in an increase in the intensity of labeling to phospho-ERK over the neuroblastic layer (Fig. 5B–D). While 0.1 mM UTP (Fig. 5D) induced an increase in phospho-ERK immunoreactivity only over the inner and outer plexiform layers of the retina, 0,5 mM ATP (Fig. 5C) induced a robust increase in the

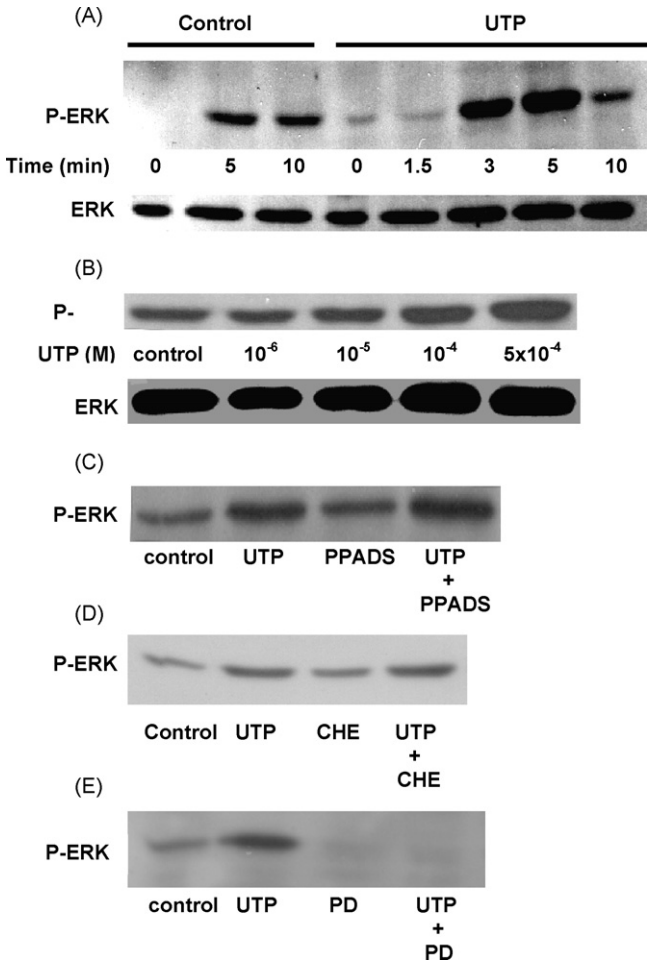

Fig. 4. Activation of the ERK pathway induced by UTP in retinas from embryos at E8. (A) Time-course of UTP-stimulated phosphorylation of ERK (UTP 0.1 mM). Note that UTP induced a transient activation of ERK pathway that peaked by 5 min of incubation. (B) Activation of ERK as a function of UTP concentration. Retinas were incubated with the agonist for 5 min. (C) Effect of the P2 antagonist PPADS on UTP-induced activation of the ERK pathway. (D, E) Effect of the MEK inhibitor, PD98059 (PD) and PKC inhibitor Chelerythrine chloride (CHE) on UTP-induced activation of the ERK pathway.

immunoreactive labeling over the neuroblastic layer and over both prospective plexiform layers. ADP also induced an increase in immunoreactivity for phosho-ERK over the neuroblastic layer, although the effect of this nucleotide was less pronounced than the effect of ATP (Fig. 5B).

In order to verify if the increase in phospho-ERK immunoreactivity over the neuroblastic layer was related to the proliferation of progenitor cells in this tissue, labeling of proliferating cells with BrdU in nucleotide stimulated explants of retinas from embryos at E8 was performed. As can be observed in Fig. 6A, the number of $BrdU^+$ cells in the neuroblastic layer increased from 100 ± 6.03 to $\sim 185.98 \pm 15.30\%$ and $\sim 190.93 \pm 8.06\%$ when retinal explants were incubated with 0.1 mM ATP or 0.1 mM ADP, for 24 h, respectively. As expected by the previous work performed in chick retinal monolayer cultures (Sanches et al., 2002), 0.1 mM UTP did not increase the number of BrdU + cells. Retinal cell progenitors positive for BrdU were localized in the neuroblastic layer of all the retinal explants (Fig. 6B–E).

ATP-induced increase in the number of BrdU + cells and in phospho-ERK immunoreactivity were observed in the neuroblastic layer of retinal explants or intact retinas, respectively. In order to verify if the ATP-induced increase in proliferation of progenitors of the neuroblastic layer was dependent on the activation of the ERK pathway, retinal explants of embryos at E7 were incubated for 24 h with 0.1 mM ATP in the presence of 50 μM PD 98059 or 20 μM U 0126, two inhibitors of the ERK signaling pathway (Fig. 7). Incorporation and labeling of BrdU + progenitor cells was performed as described in Section 2. Both compounds completely blocked the ATP-induced increase in the number of BrdU + cells in the neuroblastic layer of the explants (% of control: ATP = 191.32 ± 7.93; ATP + PD 98059 = 95.03 ± 8.02; ATP + U 0126 = 97.42 ± 8.34). Compared to control explants, no significant decrease in the number of BrdU + cells was observed in explants treated only with the inhibitors.

4. Discussion

Previously, ATP was shown to induce cell proliferation in chick retinal cells in monolayer cultures (Sanches et al., 2002; França et al., 2007). In this culture condition, reactive gliosis could be taking place as cells were dissociated prior to culturing. Since cell proliferation due to reactive gliosis was shown to occur in retinas submitted to toxic insults (Dyer and Cepko, 2000; Fisher and Reh, 2001), ATP could be inducing the proliferation of cells activated by reactive gliosis in the monolayer cultures. In the present study, we show that ATP induces an increase in $[^3H]$-thymidine incorporation and number of BrdU positive cells in retinas cultured under the form of explants. Under this condition, histotypic environment of the tissue may be well preserved, although dissection of the retinas induces the death of ganglion cells due to axotomy of their axons. However, cell proliferation induced by reactive gliosis was shown to be detected only 48 h after the treatment of retinal explants or intact retinas with reagents such as ouabain or NMDA (Dyer and Cepko, 2000; Fisher and Reh, 2001). In contrast, our data showed that treating retinal explants with ATP for 24 h already increased the incorporation of $[^3H]$-thymidine and the number of BrdU positive cells in the neuroblastic layer. Thus, our observations favor the hypothesis that this nucleotide increases the proliferation of progenitors. Accordingly, in monolayer cultures, the effect of ATP on cell proliferation occurs in tissues coming from embryos in a well defined developmental window that ranges from E6 to E9 (Sanches et al., 2002), a period where glial and bipolar cell progenitors are dividing. Our present data show that ATP induces an increase in the incorporation of $[^3H]$-thymidine in a restricted manner, during the first 72–96 h after the beginning of the explant cultures from retinas of embryos at E7. Thus, our present data support the idea that ATP only affects the proliferation of late developing progenitors of the retina, most probably glial/bipolar cell progenitors.

Stimulation of P2Y1 receptors by nucleotides induces an increase in the expression of functional ecto-ATPases (NTPDase1) in retinal pigment epithelial cells in culture (Lu

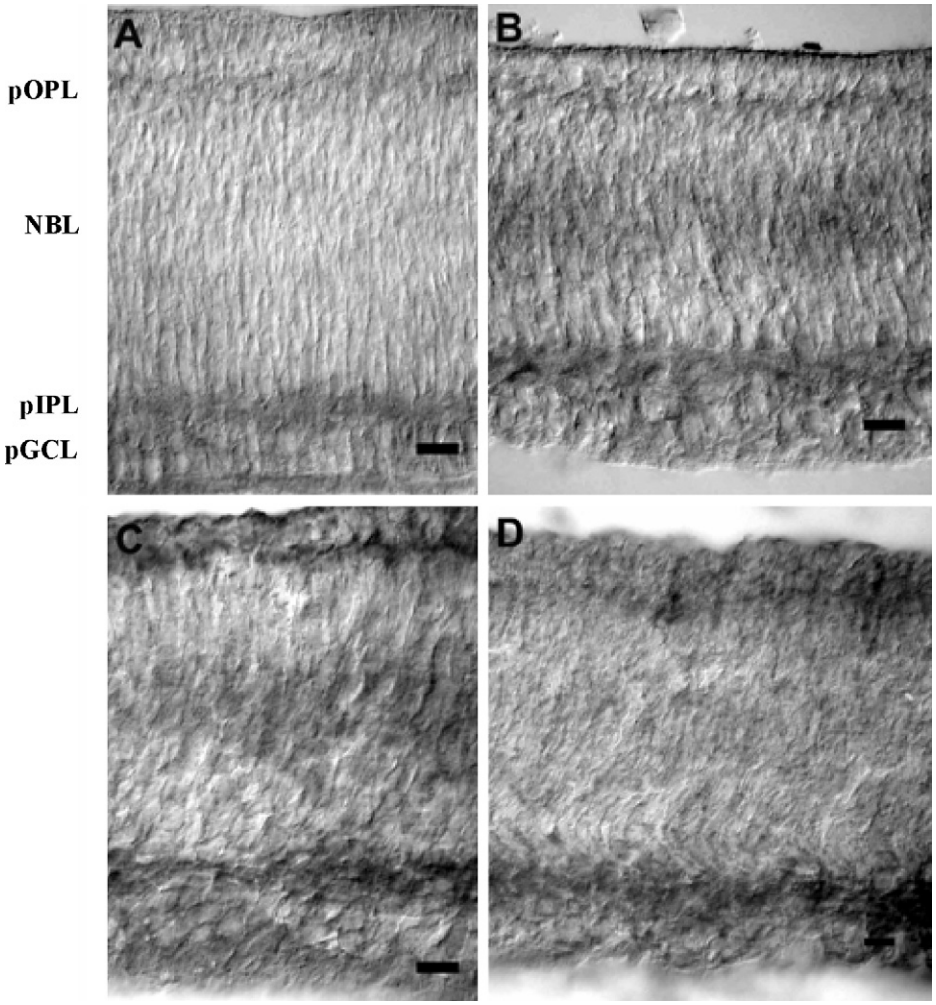

Fig. 5. Photomicrographs of phospho-ERK immunoreactivity on radial sections of the chick retinas from 8-old-day embryos. Tissues were incubated with the agonist for 5 min. (A) Control retina in BME without agonist. Note Phospho-ERK immunoreactivity in the prospective OPL, IPL. (B–D) Phospho-ERK immunoreactivity in retinas treated with: (B) 1 mM ADP; (C) 0.5 mM ATP; (D) 0.1 mM UTP. POPL, prospective outer plexiform layer; NBL, neuroblastic layer; pIPL, prospective inner plexiform layer; pGCL, prospective ganglion cell layer. Scale bar = 10 μm.

et al., 2007). Our present data showed that ATP-induced cell proliferation decreased after a 48 h incubation of the retinal explants. Thus, instead of a developmentally regulated decrease in ATP-induced proliferation, our data could reflect the expression of an ecto-ATPase that degraded the exogenous ATP added to the explants incubated for 48 h or longer periods of time. If this was the case, ATP-induced [^{3}H]-thymidine incorporation should not decrease in explants incubated in the presence of the ectonucleotidase inhibitor ARL 67156. Our data revealed that this compound did not affect the decrease in ATP-stimulated incorporation of [^{3}H]-thymidine after 48 h of incubation of the explants, suggesting that degradation of ATP added to the cultures was not responsible for the decrease in ATP-induced incorporation of [^{3}H]-thymidine after longer periods of incubation. Moreover, our data revealed that incubation of the explants with the ectonucleotidase inhibitor alone induced an increase in [^{3}H]-thymidine incorporation that was comparable to the ATP-induced [^{3}H]-thymidine incorporation. These results suggest that endogenous ATP can be released from the cells in sufficient amounts to stimulate cell proliferation in the retinal explants. In good agreement with this is the previous observation that chick embryo retinal cells in culture accumulate ATP when cultivated for 1, 24 or 48 h (Sugioka et al., 1999a,b).

Dimitropoulou and Bixby (2000) have shown, by Western blot experiments for phospho-ERKs, that in retinal preparations only one band of 43 kDa is detected. Here, we show that all nucleotides stimulated the phosphorylation of a 43 kDa ERK in retinas from chick embryos at E8, an effect that was transient, dose-dependent and blocked by the MEK 1 inhibitor PD 98059. Since, this compound and also U 0126, another inhibitor of MEK, blocked the ATP-induced BrdU labeling of proliferating cells in the neuroblastic layer of retinal explants, our results suggest that activation of the ERK pathway is a necessary step for the nucleotide induced proliferation of late developing progenitors in the neuroblastic layer of the chick retina.

The intracellular calcium concentration has a key role in CNS development and has been related to differentiation, migration and other phenomena (Pearson et al., 2002). Calcium waves are also important signals to coordinate cell cycle

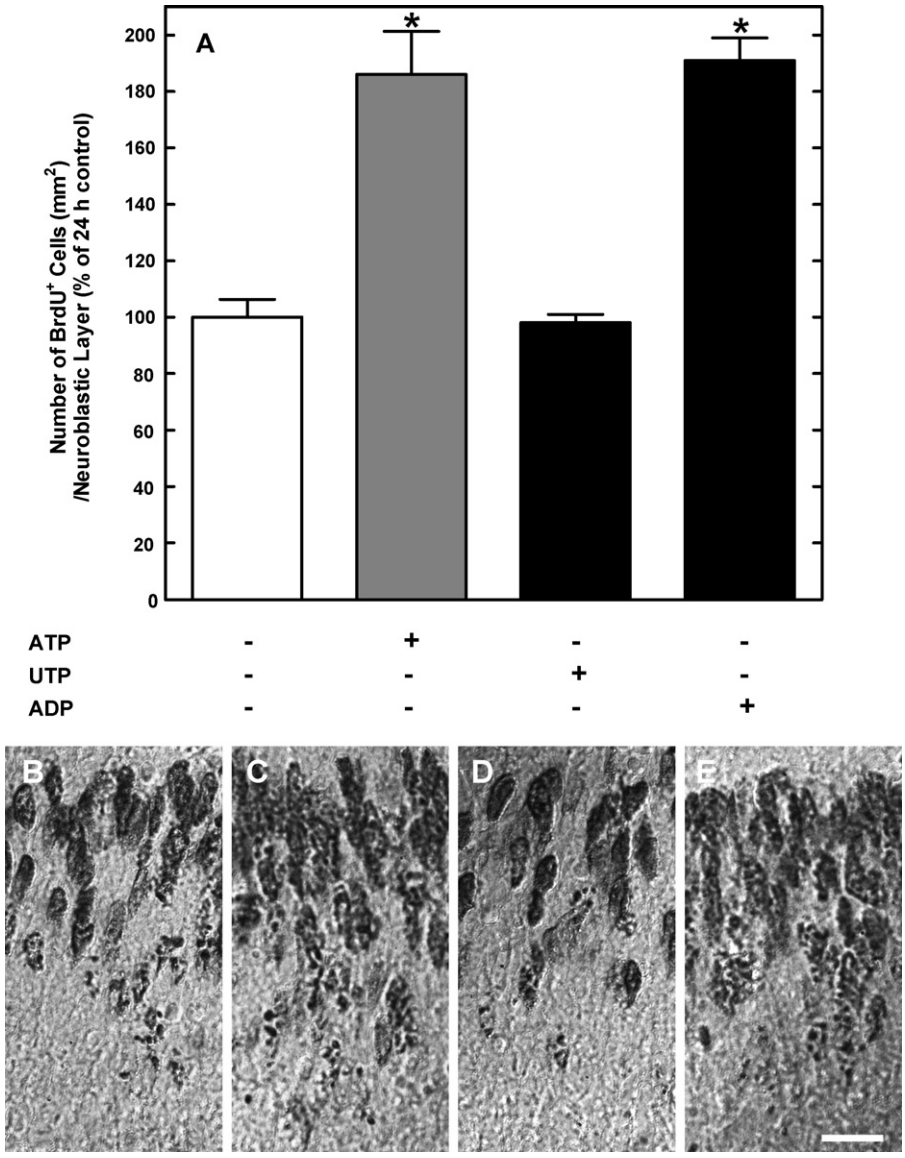

Fig. 6. Nucleotide-induced increase in the number of $BrdU^+$ cells in the neuroblastic layer of chick embryos retinal explants. Retinas from 7-day-old chick embryos were dissected, cut in ~1 mm^2 pieces and cultured as described in Section 2. ATP, UTP or ADP, at a final concentration of 0.1 mM, was added just after the beginning of the cultures. At 23 h of culture, 3 μg/mL BrdU was added, the explants incubated for an additional 1 h, washed and processed for analysis of the BrdU labeled cells. (A) Counting of BrdU + Cells (mm^2) in the neuroblastic layer as percentage of 24 h control cultures. Data represent the mean ± S.E.M. of three experiments performed in triplicate. $*p < 0.001$, as compared to control. Photomicrographs of $BrdU^+$ profiles on neuroblastic layer of chick retinas from 7-old-day embryos cultured by 24 h in control medium (B) or in the presence of 0.1 mM ATP (C), 0.1 mM UTP (D) or 0.1 mM ADP (E). Scale bar = 10 μm.

(Poenie et al., 1985; Keith et al., 1985; Kao et al., 2001; Berridge, 1995). The present work shows that either ATP, ADP or UTP were able to induce the accumulation of [^{3}H]-phosphoinositides in a dose-dependent manner. Most important, nucleotide-dependent [^{3}H]-InsPs accumulation was predominant in early developing tissues, during a developmental stage where proliferation is abundant. Since metabolism of phosphoinositides induces the mobilization of intracellular calcium, our data support that nucleotide-dependent accumulation of [^{3}H]-InsPs might be related to cell proliferation in this tissue. This idea is in good agreement with previous data showing that prolonged incubation with 2,5-di-*tert*-butylhydroquinone (DBHQ), a condition that depletes intracellular calcium stores, decreased DNA synthesis in chick retinal organ and monolayer cultures (Sugioka et al., 1999a). It also agrees with data showing that U73122, an inhibitor of phospholipase C inhibited ATP-induced cell proliferation in chick retinal cells in monolayer cultures (Sanches et al., 2002).

Neary et al. (1999) have shown that phosphorylation of ERKs in cortical astrocytes in culture is mediated by activation of a phospholipase D and a calcium-independent PKC, but not by activation of PLC or a calcium-dependent isoform of PKC, although nucleotides stimulate the formation of phosphoinositides and proliferation of these cells. Here we show that the inhibitor of PLC U73122 completely blocked the phosphorylation of ERK induced by ADP. Moreover, chelerythrine chloride, a general inhibitor of PKC also blocked the phosphorylation of ERK induced by ADP but not by UTP.

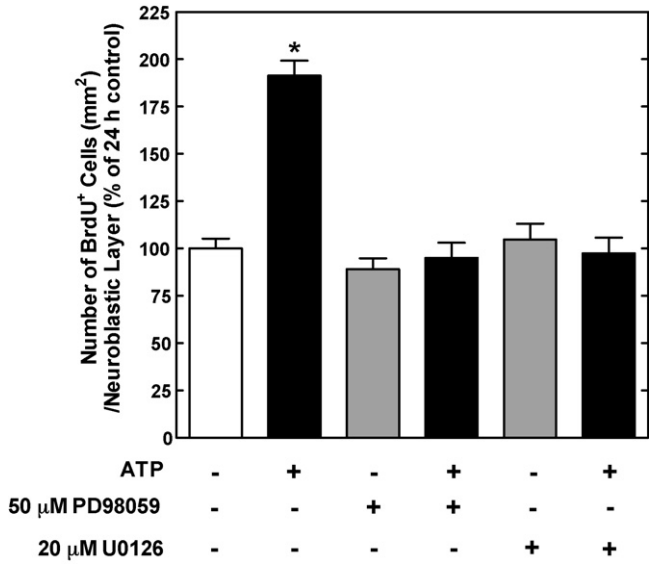

Fig. 7. Effect of ERK inhibitors on ATP-induced increase in the number of $BrdU^+$ cells in the neuroblastic layer of chick embryo retinal explants. Retinas from 7-day-old chick embryos were dissected, cut in ~1 mm^2 pieces and cultured with ATP, at a final concentration of 0.1 mM, in the presence or absence of 50 μM PD 98059 or 20 μM U0 126. At 23 h of culture, 3 μg/mL BrdU was added, the explants incubated for an additional 1 h, washed and processed for analysis of the BrdU labeled cells. Data represent the mean ± S.E.M. of three experiments performed in duplicate. $*p < 0.001$, as compared to control or ATP + ERK inhibitors.

Since cell proliferation mediated by activation of ERK signaling pathway was only induced in retinal explants from embryos at E7 by ADP and ATP, our data strongly support the idea that activation of the ERK signaling pathway leading to proliferation of late developing cells in the neuroblastic layer of retinal explants is mediated by a different pathway from the one that mediates the proliferative response of astrocytes. In chick retinal progenitors from embryos at E7, nucleotide-induced cell proliferation most probably involves a sequential activation of P2Y1 receptors, PLC, PKC and ERK.

UTP induced in a dose-dependent manner the accumulation of [^{3}H]-InsPs and phosphorylation of ERK, but did not affect the proliferation of cells in retinal explants from embryos at E7. Neither an increase in [^{3}H]-thymidine incorporation nor in the number of BrdU positive cells in the neuroblastic layer of chick retinal explants were ever detected. However, Pearson et al. (2002) have shown that this nucleotide affected cell proliferation in early developing chick retina. One possible explanation for this discrepancy is that UTP stimulated PLC and ERK pathways in other cell type progenitors distinct from the proliferating progenitors affected by ATP and ADP in retinas from embryos at E7 or E8. While proliferation of amacrine, horizontal and ganglion cell progenitors peaks around embryonic day 4, proliferation of Müller and bipolar cell progenitors peaks by the embryonic day 7 (Prada et al., 1991). Thus, it is reasonable to suppose that UTP-stimulated P2Y receptors affected the proliferation of progenitors that are dividing in a period before E7. This idea is consistent with previous observation by the same authors that the effect of UTP on cell proliferation is not detected in the retina by embryonic day 8 (Pearson et al., 2005). Moreover, our present data showed that chelerythrine chloride, an inhibitor of PKC, blocked only the activation of ERK pathway mediated by ADP-stimulated P2Y receptors but not that mediated by UTP-stimulated P2Y receptors. Since activation of both PLC and PKC was shown to be necessary for ATP-induced proliferation of retinal cells from embryos at E7 (Sanches et al., 2002) and that inhibitors of these enzymes blocked the phosphorylation of ERK induced by ADP, our data support the idea that, as opposed to ADP, the UTP-mediated activation of PLC and ERK pathway is not associated with the phenomenon of cell proliferation at this stage of the chick embryo retina development. In good agreement with this idea is our observation that only ATP and ADP, but not UTP, induced a robust increase in phospho-ERK immunoreactivity in cells located in the neuroblastic layer of retinas of embryos at E8. If PLC and ERK pathways mediate the proliferative effect of UTP in developing progenitors before E7 deserves to be investigated.

Finally, the treatment of retinal tissues with the purinergic antagonists PPADS and suramin, which were already used in previous studies (Sugioka et al., 1999b; Pearson et al., 2002; Sanches et al., 2002), completely blocked the ADP-mediated accumulation of [^{3}H]-InsPs and activation of ERK pathway and only partially the ATP-mediated responses. No significant inhibition in UTP-mediated responses was detected with these antagonists. Since both P2Y2 and P2Y4 receptors are activated by UTP and ATP, but only the functional responses mediated by P2Y2 receptors are blocked by PPADS and suramin (for review, Von Kügelgen and Wetter, 2000), our results suggest the presence in the chick retina of embryos at E8 of a P2Y4 receptor distinct from the P2Y2 receptor that was associated with cell proliferation and that was well described in earlier stages of the chick retinal development (Sugioka et al., 1996; Pearson et al., 2005). In good agreement with this idea are previous data showing the expression of mRNAs for the P2Y4 receptor subtype in the rat, human and salamander retina (Fries et al., 2004a,b, 2005).

Acknowledgements

We would like to thank Maria Leite Eduardo for technical assistance and Dr. Elizabeth Giestal Araujo e Dr. Roberto Paes de Carvalho for helpful discussions. This work was supported by grants from FAPERJ, CAPES, MCT-PRONEX, CNPQ and PROPP-UFF.

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