Ca^{2+} responses to ATP via purinoceptors in the early embryonic chick retina

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1. The action of adenosine triphosphate on cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) was studied in the retinal cell of early embryonic chicks with fura-2 fluorescence measurements. The fluorescence was measured from the whole neural retina dissected from chick embryos at embryonic day three (E3).
2. Bath application of ATP ($\geqslant$30 μM; EC_{50}, 128 μM) raised $[Ca^{2+}]_i$ by the release of Ca^{2+} from intracellular Ca^{2+} stores, since the Ca^{2+} response to ATP occurred even in a Ca^{2+}-free medium.
3. The Ca^{2+} response to ATP was mediated by P_{2U} purinoceptors. An agonist for P_{2U} purinoceptors, uridine triphosphate (UTP), evoked Ca^{2+} rises more potently ($\geqslant$3 μM; EC_{50}, 24 μM) than ATP. Agonists for P_{2X} purinoceptors, α,β-methylene ATP and β,γ-methylene ATP, or an agonist for P_{2Y} purinoceptors, 2-methylthio ATP (500 μM each), caused no Ca^{2+} response. Suramin (100 μM) and Reactive Blue 2 (50 μM) almost completely blocked the Ca^{2+} responses to 500 μM ATP and 200 μM UTP.
4. The developmental profile of the Ca^{2+} response to ATP was studied from E3 to E13. The Ca^{2+} response to ATP was largest at E3, drastically declined towards E8 and decreased further until E11–13.
5. These results suggest that the Ca^{2+} mobilization by ATP via P_{2U} purinoceptors is characteristic of early embryonic retinal cells.

Adenosine triphosphate is one of the neurotransmitters in adult nervous systems (Zimmermann, 1994). ATP also acts on embryonic or developing cells of nervous and non-nervous systems by increasing intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Release of Ca^{2+} from intracellular Ca^{2+} stores is evoked by ATP in the otocyst epithelium of the early embryonic chick (Nakaoka & Yamashita, 1995) and a mouse mesodermal stem cell line (Kubo, 1991). ATP increases inositol phosphates (Ins*P*s) levels and raises $[Ca^{2+}]_i$ in astrocytes cultured from embryonic rat spinal cord (Salter & Hicks, 1994, 1995), cultured neurones from embryonic rat brain (Mironov, 1994), myotubes cultured from embryonic chicks (Häggblad & Heilbronn, 1988), a murine myoblast cell line (Henning, Duin, den Hertog & Nelemans, 1993) and in the dissociated cells from whole early embryonic chicks (Laasberg, 1990; Lohmann, Drews, Donié & Reiser, 1991). The Ca^{2+} response to ATP in these embryonic or differentiating cells could be one of specific physiological properties of developing cells, since cellular proliferation and differentiation are regulated by Ca^{2+} mobilization (Berridge, 1993; Lauder, 1993). However, there is no systematic study of the developmental profile of the ATP-induced Ca^{2+} rise. We have investigated the action of ATP on $[Ca^{2+}]_i$ in embryonic chick neural retina during development and revealed a dramatic decline of the ATP-induced Ca^{2+} rise before synaptogenesis. We further found that the ATP-induced Ca^{2+} rise was mediated by P_{2U} purinoceptors. A preliminary report has been published as an abstract (Sugioka & Yamashita, 1994).

METHODS

Preparation

Chick embryos incubated for 3 days at 37–38 °C (E3, stage 18–19 according to Hamburger & Hamilton, 1951) were transferred to a plastic dish and killed by separating the head from the trunk with a pair of fine forceps. The optic cup was dissected out together with the lens vesicle. The diameter of the optic cup was 400 μm. The lens vesicle was removed and the neural retina (the inner wall of the optic cup) was separated from the outer wall of the optic cup (primitive pigment epithelium). At stages later than E3, chick embryos were killed by separating the head from the trunk with fine scissors in a plastic dish. The eyes were quickly enucleated and the central part of the neural retina (dorsolateral to the optic nerve head), which is developmentally the most advanced region (Prada, Puga, Pérez-Méndez, López & Ramírez, 1991), was trimmed so that the size of the retinal piece was 400 × 400 μm.

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The neural retina was loaded with fura-2 AM (10 μM) for 1 h at room temperature (~22 °C). The fura-2 AM was purchased from Dojin (Kumamoto, Japan) in a 1 mM dimethyl sulphoxide stock solution which was diluted with the normal bath solution containing (mM): NaCl, 137; KCl, 5; $CaCl_2$, 2·5; $MgCl_2$, 1; Hepes, 10; glucose, 22; buffered to pH 7·3 by adding NaOH.

The fura-2-loaded retina was transferred to a recording chamber (volume, 0·2 ml) and positioned by a hydraulic micromanipulator with the inner side up (for details, see Yamashita & Fukuda, 1993). The recording chamber was continuously perfused with the bath solution at a rate of 2 ml min^{-1} from a gravity-fed system. Experiments were performed at room temperature (~22 °C).

Fura-2 fluorescence measurement

A fluorescence measuring system (OSP-3, Olympus, Tokyo, Japan) with an inverted microscope (IMT-2, Olympus) was used. Fluorescence was excited at 340 nm (F_{340}) and 380 nm (F_{380}). Fluorescence intensities were measured by a photomultiplier through an objective lens (UVFL × 20; NA, 0·70) and a 510 nm bandpass interference filter (MF510W18, Olympus) from a circular field 50 μm in diameter which was positioned at the centre of the retina. The thickness of the retina was 40 μm at E3 and increased up to 70 μm at E13 (Coulombre, 1955). The focus level did not actually affect the fluorescence intensity as long as it was adjusted to around the middle level of the retina. When F_{340} increases and

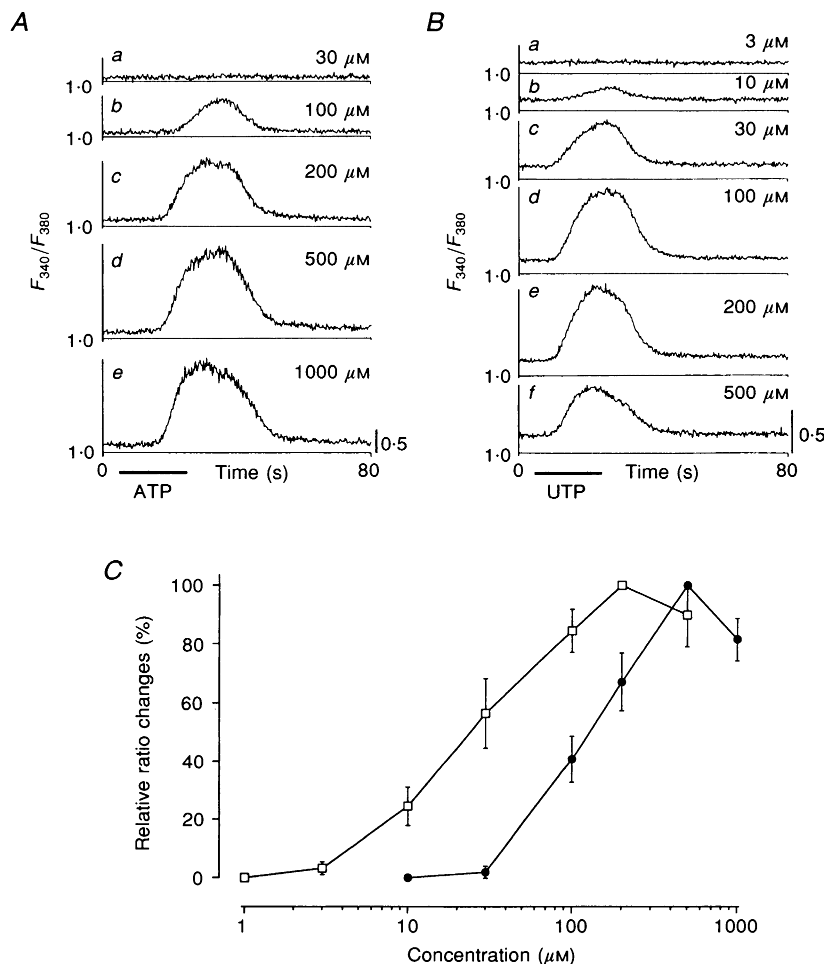

Figure 1. Dose–response relationships of Ca^{2+} responses to ATP and UTP in E3 chick retinas

A, fluorescence responses of a neural retina dissected from an embryonic day 3 (E3) chick to the bath applications of adenosine triphosphate at the concentrations indicated on the right of *Aa–e*. The test solutions were applied in this order at 7 min intervals. The duration of ATP application (20 s) is indicated by the bar. The retina was loaded with fura-2 AM. Fluorescence was excited at 340 (F_{340}) and 380 nm (F_{380}). The ratio of the two fluorescence intensities (F_{340}/F_{380}) is shown on the ordinate on a common scale given at the bottom of the right-hand side. The bath solutions contained 2·5 mM Ca^{2+}. *B*, fluorescence responses of an E3 chick retina to the bath applications of uridine triphosphate (UTP) at the concentrations indicated on the right of *Ba–f*. The test solutions were applied in this order at 7 min intervals. The duration of UTP application (20 s) is indicated by the bar. The bath solutions contained 2·5 mM Ca^{2+}. *C*, the concentration of ATP (●; $n = 11$) and UTP (□; $n = 7$) applied to E3 chick retinas (abscissa) *versus* the mean percentage of the relative change in fluorescence ratio with reference to the response to 500 μM ATP or 200 μM UTP (ordinate). Error bars indicate S.D. Two of the eleven retinas responded to 30 μM ATP by 1·9 ± 2·1 %. Three of the seven retinas responded to 3 μM UTP by 3·2 ± 2·2 %. All records were taken in the normal bath solution containing 2·5 mM Ca^{2+}.

F_{380} decreases, the ratio of F_{340}/F_{380} indicates a rise in $[Ca^{2+}]_i$ (Grynkiewicz, Poenie & Tsien, 1985). We present the fluorescence ratio to describe the relative changes in $[Ca^{2+}]_i$ without conversion to absolute values of intracellular free Ca^{2+} concentrations. The details of fluorescence measurements are described in Yamashita & Fukuda (1993).

Application of test solutions

Bath solutions were changed from the normal bath solution to a test solution for 20 s by using multi-solenoid manifold valves (General Valve Corp., Fairfield, NJ, USA). Adenosine triphosphate (ATP) (magnesium salt), caffeine, adenosine, α,β-methylene ATP (lithium salt), β,γ-methylene ATP (sodium salt), uridine triphosphate (UTP) (sodium salt) and Reactive Blue 2 were purchased from Sigma. 2-Methylthio ATP (sodium salt) was purchased from Research Biochemicals. Suramin was purchased from Wako (Osaka, Japan). A UTP concentration of 500 μM did not change the pH of the bath solution but 500 μM ATP lowered the pH from 7·3 to 7·15 and 1 mM ATP lowered the pH from 7·3 to 7·0. The effect of the decrease in pH was checked by comparing Ca^{2+} responses with 500 μM ATP at pH 7·15 and 7·3 (re-adjusted by adding NaOH), but no difference was found (tested in 4 retinas). The decrease in pH from 7·3 to 7·0 alone caused no Ca^{2+} response (tested in 11 retinas). To maintain the concentration of free Mg^{2+}, ATP in magnesium salt was used for making test solutions containing ATP. A Ca^{2+}-free bath solution was made by replacing Ca^{2+} with Na^+ and adding 1 mM EGTA.

Data presentation

Statistical comparisons were made using Student's two-tailed t test, with measurements being given as means $\pm$ S.D., and the level of significance was determined as $P < 0{\cdot}05$.

RESULTS

Dose–response relationships of Ca^{2+} responses to ATP and UTP in E3 chick retina

Bath applications of 500 μM ATP caused increases in F_{340} and decreases in F_{380} indicating rises in $[Ca^{2+}]_i$ in all the embryonic day 3 (E3) chick retinas tested ($n = 60$). Figure 1A shows typical responses of an E3 chick retina to bath applications of 30, 100, 200, 500 and 1000 μM ATP for 20 s. When 30 μM ATP was applied, no change was observed in the fluorescence ratio (F_{340}/F_{380}; Fig. 1A a). The application of 100 μM ATP evoked a clear increase in the ratio (Fig. 1A b). The Ca^{2+} response became larger with the incremental increase of ATP concentration up to 500 μM (Fig. 1A c and d). Neither the additional increase in the peak response amplitude nor the reduction in the onset latency was observed with the application of 1000 μM ATP (Fig. 1A e).

The dose–response relationship between ATP concentration (10–1000 μM) and peak amplitude of fluorescence ratio change ($\Delta F_{340}/F_{380}$) was measured in eleven E3 chick retinas. The relative amplitude of $\Delta F_{340}/F_{380}$ at each ATP concentration was plotted against the response to 500 μM ATP (Fig. 1C, ●). Application of 10 μM ATP caused no change in $[Ca^{2+}]_i$ ($n = 11$). Application of 30 μM ATP caused small Ca^{2+} responses in two of the eleven retinas, whereas 100 μM ATP evoked clear rises in $[Ca^{2+}]_i$ in all the retinas tested. The Ca^{2+} response became larger in a dose-dependent manner up to 500 μM ATP. The half-maximal response dose (EC_{50}) was 128 μM. We used 500 μM ATP to describe the characteristics of ATP response in the following sections.

Uridine triphosphate, an agonist of ATP (described in the later part of Results), also caused Ca^{2+} rises (Fig. 1B) and the dose–response relationship to UTP was measured in seven E3 chick retinas (Fig. 1C, □). Ca^{2+} rises were evoked from 3 μM of UTP in three of the seven retinas and the response was saturated at 200 μM of UTP with EC_{50} of 24 μM.

ATP caused Ca^{2+} release from intracellular Ca^{2+} stores in E3 chick retina

We examined whether the Ca^{2+} rise induced by ATP was due to Ca^{2+} influx or Ca^{2+} release by eliminating extracellular Ca^{2+} from the bath solution (Fig. 2). The Ca^{2+} response to 500 μM ATP could be evoked even in the Ca^{2+}-free medium in all the retinas tested ($n = 11$). The mean response amplitude of the eleven retinas to the first application of

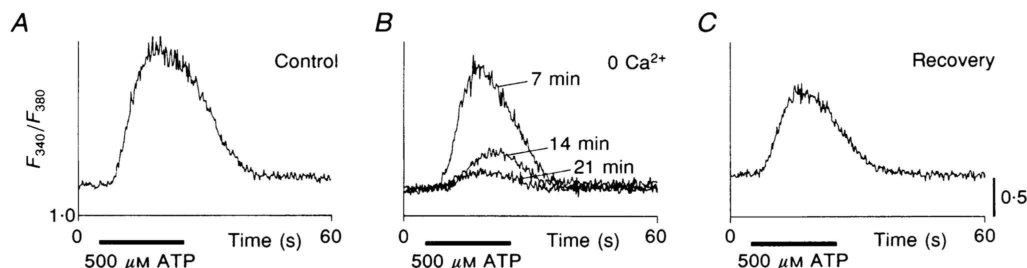

Figure 2. Effects of eliminating extracellular Ca^{2+} on the Ca^{2+} response to ATP in an E3 chick retina

A Ca^{2+} response to 500 μM ATP was recorded in the normal bath solution as a control response (A). The records in B were taken 7, 14 and 21 min after changing the bath solutions from the normal bath solution to a Ca^{2+}-free medium. The recovery control was taken 10 min after external Ca^{2+} was re-introduced (C). The normal bath solution contained 2·5 mM Ca^{2+}. In the Ca^{2+}-free medium, Ca^{2+} was replaced with Na^+ and 1 mM EGTA was added. The test solution containing ATP in B was also Ca^{2+} free. The duration of ATP application (20 s) is indicated by the bars.

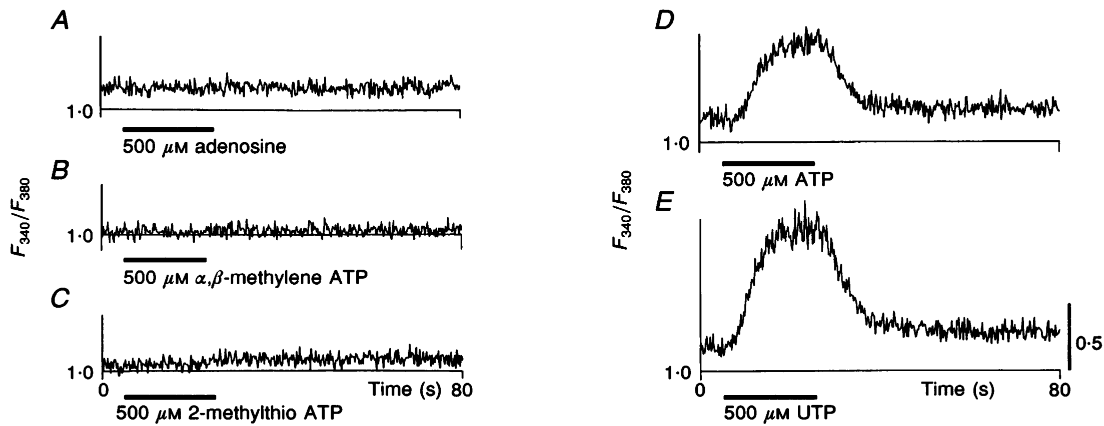

Figure 3. Ca^{2+} responses to purinoceptor agonists in an E3 chick retina

Adenosine (*A*), α,β-methylene ATP (*B*), 2-methylthio ATP (*C*), ATP (*D*) and UTP (*E*) were applied to an E3 chick retina. These agonists were applied at 500 μM in the order of *B*, *C*, *A*, *D* and *E* at 6 min intervals. The duration of agonist application (20 s) is indicated by the bars. All records were taken in the normal bath solution containing 2·5 mM Ca^{2+}.

ATP in the Ca^{2+}-free medium was almost the same (97·5 ± 13·1 %, $n = 11$) as that in the normal bath solution which contained 2·5 mM Ca^{2+}. However, the Ca^{2+} response to successive ATP applications in the Ca^{2+}-free medium showed a marked decline (Fig. 2*B*). When the normal bath solution was re-introduced (Fig. 2*C*), the Ca^{2+} response to ATP was restored. These results suggested that the release of Ca^{2+} from intracellular Ca^{2+} stores was evoked by extracellular ATP and the Ca^{2+} store could be depleted in the absence of extracellular Ca^{2+}.

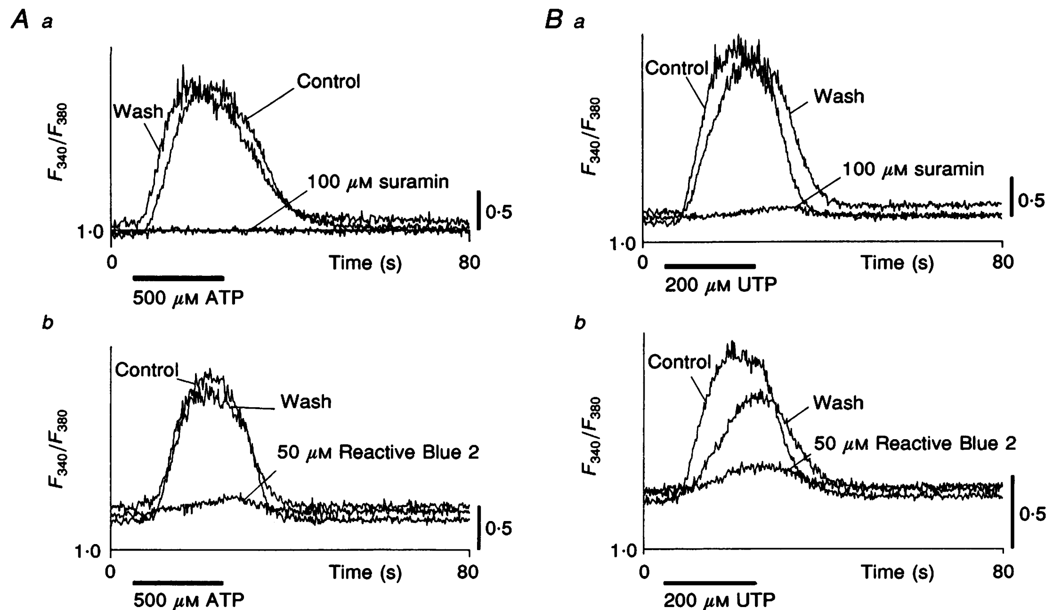

Figure 4. Effects of P_2 purinoceptor antagonists on the Ca^{2+} responses to ATP and UTP in E3 chick retinas

A, the effects of suramin (100 μM; *A a*) and Reactive Blue 2 (50 μM; *A b*) on the response to 500 μM ATP. The records in the presence of suramin or Reactive Blue 2 were taken 7 min after changing the bath solutions to the antagonist-containing medium. The recovery controls were taken after washing suramin for 7 min or Reactive Blue 2 for 25 min. The duration of ATP application (20 s) is indicated by the bars. All records were taken in the bath solutions containing 2·5 mM Ca^{2+}. *B*, the effects of suramin (100 μM; *Ba*) and Reactive Blue 2 (50 μM; *B b*) on the response to 200 μM UTP. The records in the presence of suramin or Reactive Blue 2 were taken 7 min after changing the bath solutions to the antagonist-containing medium. The recovery controls were taken after washing suramin for 7 min or Reactive Blue 2 for 15 min. The duration of UTP application (20 s) is indicated by the bars. All records were taken in the bath solutions containing 2·5 mM Ca^{2+}.

We applied caffei.. (an activator of ryanodine-sensitive Ca^{2+} stores) at 5 and 10 mM to five E3 chick retinas for 20 s in the normal bath solution. However, no Ca^{2+} rise was observed (data not shown).

Involvement of P_{2U} purinoceptors

Adenosine (agonist for P_1 purinoceptors) at 500 μM was tested in three E3 retinas but no Ca^{2+} response was observed (Fig. 3*A*). Agonists for P_{2X} purinoceptors, α,β-methylene ATP and β,γ-methylene ATP, and an agonist for P_{2Y} purinoceptors, 2-methylthio ATP, were tested at a concentration of 500 μM in E3 chick retinas. However, no Ca^{2+} response was evoked by α,β-methylene ATP (Fig. 3*B*; $n = 2$), β,γ-methylene ATP (data not shown), or 2-methylthio ATP (Fig. 3*C*; $n = 4$). An agonist for P_{2U} purinoceptors, UTP (500 μM), evoked a larger Ca^{2+} response (Fig. 3*E*) than the response to ATP at the same concentration (Fig. 3*D*). The mean response amplitude to 500 μM UTP was 132·5 ± 8·8% ($n = 5$) with reference to the response to 500 μM ATP. The dose–response relationships in Fig. 1*C* indicated that UTP was more potent than ATP in evoking Ca^{2+} rises.

The Ca^{2+} response to ATP (500 μM) was blocked by suramin (100 μM; Fig. 4*A a*) and Reactive Blue 2 (50 μM; Fig. 4*A b*). Suramin at 100 μM completely blocked the Ca^{2+} response to 500 μM ATP ($n = 5$). The peak amplitude of the increase in F_{340}/F_{380} caused by 500 μM ATP was inhibited by 90·8 ± 3·4% ($n = 7$) in the presence of 50 μM Reactive Blue 2. The Ca^{2+} response to UTP (200 μM) was also blocked by suramin (100 μM; Fig. 4*Ba*) and Reactive Blue 2 (50 μM; Fig. 4*Bb*). The peak amplitude of the increase in F_{340}/F_{380} caused by 200 μM UTP was inhibited by 86·5 ± 3·1% ($n = 3$) in the presence of 100 μM suramin and by 83·6 ± 5·4% ($n = 5$) in the presence of 50 μM Reactive Blue 2.

Developmental changes in the Ca^{2+} response to ATP

Developmental changes in the Ca^{2+} response to ATP were examined from E3 to E13. Typical records are shown in Fig. 5*A* on a common scale to compare the amplitude of the Ca^{2+} response at each embryonic day. The Ca^{2+} response was largest at E3. At E5, the response became smaller in the peak amplitude. Then the Ca^{2+} response drastically declined from E5 to E8 and became increasingly smaller at E11 and E13. The basal levels of F_{340}/F_{380} were variable around 1·0 depending on the retinas tested but we could not find any consistent effect of these variances on the amplitude of Ca^{2+} responses to ATP. No systematic change in the onset latency was observed during the course of the development we examined.

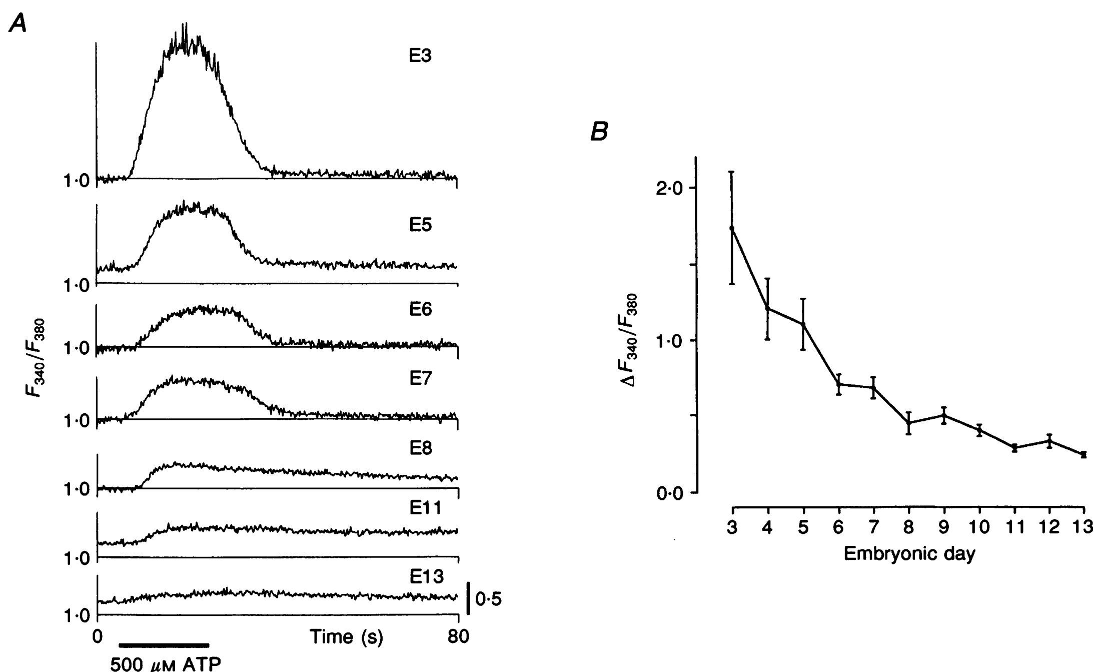

Figure 5. Developmental changes in the Ca^{2+} response to ATP from E3 to E13

A, 500 μM of ATP was applied during the period (20 s) indicated by the bar. The fluorescence ratio (F_{340}/F_{380}) is shown on the ordinate on a common scale given at the bottom of the right-hand side. Records were taken from different chick retinas on the embryonic days indicated. The bath solution contained 2·5 mM Ca^{2+}. *B*, peak amplitude of the increase in the fluorescence ratio ($\Delta F_{340}/F_{380}$) to 500 μM ATP (ordinate) *versus* embryonic day of the retina tested (abscissa). Each point indicates the mean $\Delta F_{340}/F_{380}$ recorded from the retinas of the following numbers: $n = 13$ (E5), 12 (E3), 11 (E4), 8 (E6, 7), 6 (E8, 9, 11, 12), 5 (E10) and 4 (E13). Error bars indicate S.D. All records were taken in the normal bath solution containing 2·5 mM Ca^{2+}.

The peak amplitude of the increase in the fluorescence ratio ($\Delta F_{340}/F_{380}$) was plotted as a function of embryonic day in Fig. 5*B*. From E3 to E5, the mean $\Delta F_{340}/F_{380}$ decreased significantly from 1·74 to 1·10 ($P < 0·05$). The Ca^{2+} response continued to decrease towards E8, when the mean $\Delta F_{340}/F_{380}$ became 0·44. A further decline was observed from E8 to E11 ($\Delta F_{340}/F_{380}$ at E11, 0·28), whereas no statistical difference was observed between the response amplitudes at E11 and E13 ($P > 0·1$).

When the decay phases of the fluorescence responses at E3–7 and E8–13 were compared (Fig. 5*A*), the decay was slower in the latter group, raising the possibility that a persistent component was added to the Ca^{2+} response during development. We examined this point first by eliminating extracellular Ca^{2+} and then pharmacologically.

When 500 μM ATP was applied to an E11 chick retina, a Ca^{2+} rise occurred in the absence of extracellular Ca^{2+} (Fig. 6*A b*), but the time to peak became longer and the tail of the response returned faster to the baseline in the Ca^{2+}-free medium compared with the responses in the normal bath solution (Fig. 6*A a* and *c*). The mean peak response amplitude of the E11 chick retina decreased to 71·0 ± 13·1% ($n = 5$) by the elimination of extracellular Ca^{2+}. These effects of the removal of extracellular Ca^{2+} suggested that the rise in $[Ca^{2+}]_i$ by ATP was due to both Ca^{2+} release and Ca^{2+} influx at the late embryonic stage (E11).

Adenosine triphosphate (500 μM) evoked intracellular Ca^{2+} rises in the presence of 100 μM suramin (Fig. 6*B b*) in the two E9 retinas tested, where the time to peak became longer and the tail of the response returned faster to the baseline. Reactive Blue 2 (50 μM) had no effect (Fig. 6*Bd*) on either of the two retinas. Thus, differences in antagonist properties were noticed between E3 and E9 retinas. α,β-Methylene ATP, β,γ-methylene ATP, or 2-methylthio ATP did not show any Ca^{2+} response (data not shown), whereas UTP still caused a comparable Ca^{2+} response (Fig. 6*Bf*) with those to ATP at the same concentration (500 μM; Fig. 6*Ba*, *c* and *e*).

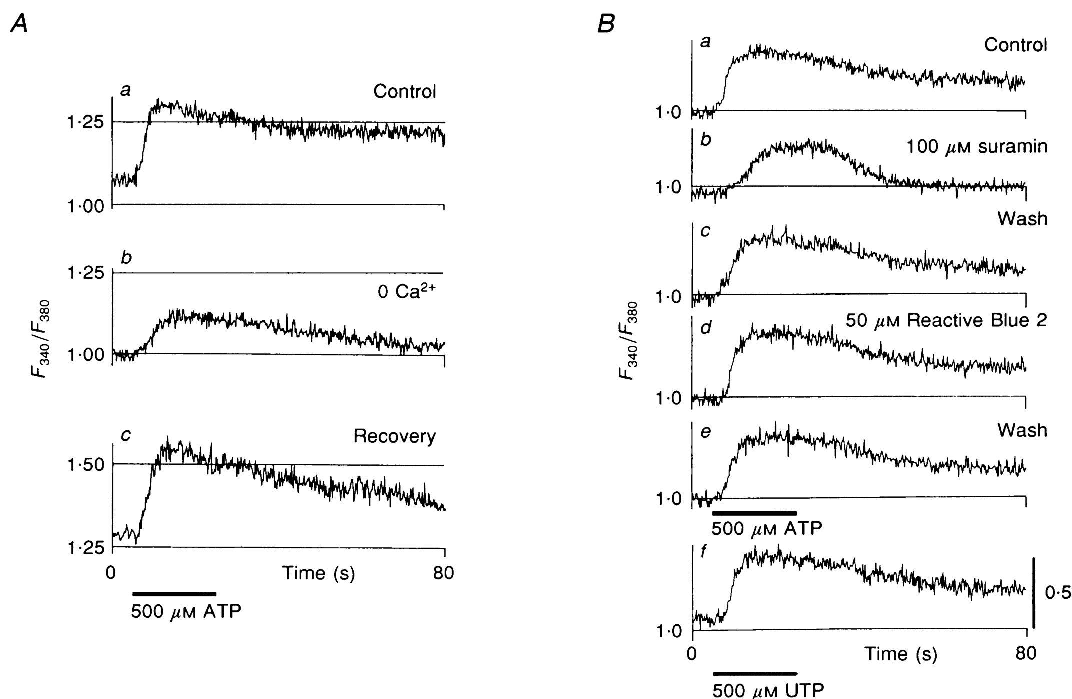

Figure 6. Ca^{2+} responses to ATP in E9 and E11 chick retinas

A, effect of eliminating extracellular Ca^{2+} on the Ca^{2+} response to 500 μM ATP in an E11 chick retina. The control record was taken in the normal bath solution (*A a*). The record taken 10 min after changing the bath solutions to a Ca^{2+}-free medium is shown in *A b*. The recovery control was taken 9 min after external Ca^{2+} was re-introduced (*A c*). The normal bath solution contained 2·5 mM Ca^{2+}. In the Ca^{2+}-free medium, Ca^{2+} was replaced with Na^+ and 1 mM EGTA was added. The test solution containing ATP in *A b* was also Ca^{2+} free. The duration of ATP application (20 s) is indicated by the bar. *B*, effects of P_2 purinoceptor antagonists on the Ca^{2+} response to 500 μM ATP and a Ca^{2+} response to UTP in an E9 chick retina. *Ba*, the control response to 500 μM ATP in the normal bath solution. *Bb*, record taken 8 min after changing the bath solutions to a medium containing suramin (100 μM). *Bc*, the recovery control after washing suramin for 7 min. *Bd*, record taken 12 min after changing the bath solutions to a medium containing 50 μM Reactive Blue 2. *Be*, the recovery control after washing Reactive Blue 2 for 7 min. *Bf*, response to 500 μM UTP, 9 min after recording *Be*. The duration of ATP or UTP application (20 s) is indicated by the bars. All records were taken in the bath solutions containing 2·5 mM Ca^{2+}.

DISCUSSION

Our study demonstrates that ATP raises $[Ca^{2+}]_i$ in early embryonic chick retinas via P_{2U} purinoceptors and that the Ca^{2+} response drastically declines before synaptogenesis. We discuss the mechanism for the intracellular Ca^{2+} rise and the developmental change in the ATP response.

Mechanisms for purinergic Ca^{2+} rise in the E3 chick retina

Uridine triphosphate, an agonist for P_{2U} purinoceptors (Dubyak & El-Moatassim, 1993), was more effective than ATP to evoke Ca^{2+} rises. Antagonists for P_2 purinoceptors, suramin and Reactive Blue 2, blocked the Ca^{2+} responses to ATP and UTP. From these pharmacological properties it could be concluded that P_{2U} purinoceptors were responsible for the Ca^{2+} response to ATP in the E3 chick retina.

Adenosine triphosphate caused Ca^{2+} rises in the Ca^{2+}-free bath solution to the same extent as those in the presence of external Ca^{2+} (2·5 mM). This strongly suggests that the release of Ca^{2+} from intracellular Ca^{2+} stores was evoked by ATP. This suggestion may be compatible with the above pharmacological conclusion, because the P_{2U} purinoceptor is regarded as one of G protein-coupled receptors, which produce inositol 1,4,5-trisphosphate (InsP_3) to induce Ca^{2+} release from InsP_3-sensitive Ca^{2+} stores (Dubyak & El-Moatassim, 1993). The fact that Li^+ enhances the ATP-induced Ca^{2+} rise in the E3 chick retina (Sakaki & Yamashita, 1995) may support the involvement of InsP_3, since Li^+ inhibits hydrolysis of Ins$P$s (Berridge, Downes & Hanley, 1982). It has been reported that the accumulation of InsP_3 is evoked by ATP in dissociated cells from chick embryos (Laasberg, 1990; Lohmann *et al.* 1991). Thus InsP_3-sensitive Ca^{2+} stores could be involved in the Ca^{2+} release by ATP. Another group of intracellular Ca^{2+} stores, namely ryanodine-sensitive Ca^{2+} stores, can be activated by caffeine (Miller, 1991). However, no Ca^{2+} response was evoked by caffeine in our investigation. It should also be kept in mind that our experiments were made at room temperature (~22 °C) at which metabolic activities would be lower than those at normal incubation temperature.

Developmental changes in the Ca^{2+} response to ATP

The Ca^{2+} response to ATP was largest at E3, drastically declined towards E8 and decreased further until E11–13, before the stage of synaptogenesis (E14; Sheffield & Fischman, 1970). The decline in $\Delta F_{340}/F_{380}$ might be due to an increase in the thickness of the retina. F_{340} and F_{380} could be differentially absorbed depending on the thickness of the retina so that F_{340} decreases and F_{380} increases as the thickness of the retina increases. However, the basal level of F_{340}/F_{380} did not decrease during the development of the retina, indicating that such differential absorption is not underlying the decline in the Ca^{2+} response to ATP. Alternatively, the decline might be explained by a decrease in responding cell number within the measurement area. However, the Ca^{2+} response to ATP declines from E3 when the retinal cells are actively proliferating (Prada *et al.* 1991). The decline of ATP response may be due to the decrease in the number of purinoceptors, although we did not study the number of purinoceptors quantitatively. The production of the second messenger InsP_3 as a candidate, could be decreased during development. The muscarinic Ca^{2+} response, which is also likely to be mediated by InsP_3, abruptly declines after E5 in the chick retina (Yamashita, Yoshimoto & Fukuda, 1994). Calvet & Ventura (1995) have shown that the accumulation of Ins$P$s by the activation of muscarinic receptors is highest at the early stages they examined (E8–14) and decreases afterwards until postnatal day 7. They suggest that this decrease could be due to structural changes in muscarinic receptors and a functional change in the receptor–effector coupling (Calvet & Ventura, 1995). The changes in the InsP_3 production and also in the number of InsP_3 receptors remain to be investigated to explain the decline of ATP-induced Ca^{2+} rise.

The pharmacological properties of the purinergic Ca^{2+} response at E9 differed from those at E3. Since UTP was still an effective agonist at E9, it could be assumed that P_{2U} purinoceptors were also present at E9. However, ATP could evoke Ca^{2+} rises in the presence of suramin and Reactive Blue 2. The antagonistic sites for these ligands at P_{2U} purinoceptors may change structurally during development. Nevertheless, a partial blockage was caused by suramin in the ATP response at E9; the rising phase became slower and the decay phase returned to the baseline faster. The partial blockage by suramin could occur at P_{2X}-like purinoceptors because suramin is antagonistic for P_{2X} purinoceptors as well as other P_2 purinoceptors, while Reactive Blue 2 is not antagonistic for P_{2X} purinoceptors (Burnstock, 1991; Barnard, Burnstock & Webb, 1994). The characteristic short time to peak and long tail of ATP responses at the later stages than E7 would be mediated by the P_{2X}-like purinoceptor which causes the influx of external Ca^{2+}. UTP could also cause these response characteristics in accordance with the fact that UTP also acts at P_{2X} purinoceptors (von Kügelgen, Bültmann & Starke, 1990; Abbracchio & Burnstock, 1994). The fact that the ATP-induced Ca^{2+} rise was reduced by the elimination of extracellular Ca^{2+} at the late stage (E11) suggests that the contribution of Ca^{2+} influx to the purinergic Ca^{2+} rise becomes considerable during the course of development.

The action of ATP on developing cells

Trophic actions of ATP have been reported in cultured cells (Burnstock, 1993; Boarder, Weisman, Turner & Wilkinson, 1995). ATP stimulates mitotic activities in vas deferens tumor cell line (Huang, Wang & Heppel, 1989), cultured vascular smooth muscle cells (Erlinge, Yoo, Edvinsson, Reis & Wahlestedt, 1993) and cultured astrocytes (Rathbone *et al.* 1992; Abbracchio, Saffrey, Höpker & Burnstock, 1994; Neary, Whittemore, Zhu, Norenberg, 1994). ATP induces intracellular Ca^{2+} rises by Ca^{2+} release in astrocytes cultured from embryonic rat spinal cord (Salter & Hicks, 1994, 1995), embryonic chick otocyst epithelium (Nakaoka & Yamashita, 1995) and also in other differentiating cells (Hägglad &

Heilbronn, 1988; Laasberg, 1990; Kubo, 1991; Lohmann *et al.* 1991; Henning *et al.* 1993). Such Ca^{2+} mobilization caused by ATP could be an underlying mechanism for the mitogenic action of ATP, since intracellular Ca^{2+} plays important roles in cellular proliferation (Berridge, 1993; Lauder, 1993). For the development of chick embryos ATP could act as a trophic factor. This idea might be supported by our preliminary experiments by injecting Reactive Blue 2 into early embryonic chicks, where severe effects were observed on embryogenesis (our unpublished observations).

We demonstrated the ATP-induced Ca^{2+} mobilization in the early embryonic neural retina and showed the rapid decline of the ATP-induced Ca^{2+} response before synapse formation (E14; Sheffield & Fischman, 1970). This developmental profile may correlate with the mitotic activity of the retinal cells. Prada *et al.* (1991) have studied the process of retinal cell mitosis in the chick. The earliest cells (ganglion cells) start leaving the cell cycle at around E2, and all ganglion, horizontal and photoreceptor cells are postmitotic at E7, amacrine cells at E9, with bipolar and Müller cells at E12. The time course of accumulation of born retinal cells (Prada *et al.* 1991) is in a reverse relationship with the time course of the Ca^{2+} response to ATP. The decrease in the mitotic activities of these retinal cells might, therefore, be related to the decline of the ATP-induced Ca^{2+} mobilization.

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