Ins(1,4,5)P_3 formation and fluctuating chloride current response induced by external ATP in *Xenopus* oocytes injected with embryonic guinea pig brain mRNA

Franck Fournier, Eric Honoré, Thibault Collin and Pierre Guilbault

Laboratoire de Physiologie Cellulaire, UFR de Biologie, Université des Sciences et Techniques de Lille-Flandres-Artois, 59655 Villeneuve d'Asq Cédex, France

Received 25 September 1990; revised version received 29 October 1990

In voltage-clamped *Xenopus* oocytes injected with embryonic guinea pig mRNA, effective concentrations of extracellular ATP elicited an inward fluctuating current. This current, carried by Cl-ions, was mainly dependent upon liberation of Ca^{2+} ions from stores as demonstrated by experiments using intracellular EGTA loading and TMB-8 superfusion. Neomycin inhibited these fluctuating currents indicating that the transplanted purinoceptor is linked to phospholipase C activity and triggers Ins(1,4,5)P_3 formation. Ins(1,4,5)P_3 production evoked by external ATP was clearly demonstrated by directly measuring the water-soluble Ins(1,4,5)P_3 level in injected oocytes. Finally, it is suggested that the ATP effect was mediated by a Ca^{2+} release from Ins(1,4,5)P_3 sensitive pools since heparin blocked the ATP responsiveness. The acquired purinoceptor may be made apparent to a P_2 subtype since ATP and ADP were equipotent in eliciting Cl^- current while AMP and Adenosine were ineffective in injected oocytes.

Xenopus oocyte; Guinea pig brain mRNA; Ins(1,4,5)P_3; Internal Ca^{2+} pool; Extracellular ATP; P_2 purinoceptor

1. INTRODUCTION

The close association between neuronal activation and ATP secretion indicates that extracellular ATP can act as a cotransmitter [1] and exert a feedback modulation of the transmitter release in central and peripheral nervous systems. Thus, it was previously stated that afferent fibres may utilize ATP to excite neurons in the sensory regions of the brain, as well as in the spinal cord [2-4]. Furthermore, other findings have pointed out a release of ATP from cholinergic nerve endings in the central nervous system [5]. P_2 purinergic receptors, reported to be sensitive to ATP and ADP [6], exhibit a wide variety of transduction mechanisms (Ins(1,4,5)P_3-induced Ca^{2+} mobilization, Ca^{2+} influx through receptor-operated channel). These mechanisms are not yet clearly defined but both contribute to increasing the free intracellular Ca^{2+} level. The complexity and diversity of these transduction processes led us to use the *Xenopus* oocyte translation system to express P_2 purinoceptor from embryonic guinea pig mRNA. *Xenopus* oocyte is a useful tool in the dissection of synaptic communication processes since it faithfully synthesises and assembles a variety of biologically active neurotransmitter receptors after injection of exogenous mRNA [7]. With this system, a range of studies on receptor function is accessible when the receptors are active in controlled situations. In many cases, the agonist-induced responses were detected as changes in chloride conductance as a result of the activation of endogenous Ca^{2+}-dependent Cl-channels in the oocyte plasma membrane [8]. In the present study, a description of an acquired P_2 purinoceptor activity is presented and evidence is provided for the participation of phosphoinositide breakdown and an Ins(1,4,5)P_3-induced Ca^{2+}-release from intraoocyte stores to the purinergic response.

Correspondence address: P. Guilbault, Laboratoire de Physiologie Cellulaire, UFR de Biologie, Université des Sciences et Techniques de Lille-Flandres-Artois, 59655 Villeneuve d'Asq Cédex, France

2. MATERIALS AND METHODS

Total RNA was extracted from embryonic guinea pig brain following the Chirgwin procedure [9] and poly(A)$^+$ mRNA was selected with two runs of oligo(dT) cellulose chromatography. Oocytes were dissected away from tricaine methane sulphonate (MS 222)-anesthetized adult *Xenopus laevis* and prepared as reported elsewhere [10]. Stage VI oocytes were pressure-injected with 50 ng of mRNA and incubated in a modified Barth solution (MBS) supplemented with 50 μg/ml gentamycin for 3-6 days. Uninjected oocytes served as control.

Electrophysiological measurements were performed using the two-electrode voltage-clamp technique and membrane currents were monitored on a flat-bed single-pen chart recorder. In all experiments, the holding voltage was set to −70 mV and oocytes were routinely tested in the conventional bathing medium ND96 (in mM: NaCl 96; $MgCl_2$ 2; $CaCl_2$ 1.8; Hepes 5, pH 7.4 with NaOH) unless otherwise stated. Drugs were applied either externally by addition to the superfusate or internally by pressure injection using an additional micropipette (3-8 μm in diameter). The injected volume was 0.5-0.8% of the cell volume and all injection solutions were made up

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

in 10 mM Hepes-KOH (pH 7). Experiments were conducted using batches of oocytes derived from three different donors.

To determine Ins(1,4,5)P_3 level, 40–50 injected oocytes were incubated for 24 h at 22°C with MBS supplemented with 10 μCi/ml of myo-[2-^{3}H]inositol. After extensive washing, cells were preincubated for 20 min with 10 mM LiCl in MBS medium and stimulated for 2 min with 300 μM ATP or 10 μM ACh. RNA-injected oocytes with no agonist stimulation served as control. The reaction was stopped by the addition of perchloric acid to 0.5 M and immediate homogeneisation. Subsequently, cell extracts were neutralized with 1.5 M KOH in 75 mM Hepes and applied to Dowex AG 1 × 8 columns (formate form). A stepwise elution was performed using 0.1 M formic acid and increasing concentrations of ammonium formate [11]. The radioactivity of the fraction eluted between 0.4 and 1 M ammonium formate containing Ins(1,4,5)P_3 was counted in a liquid scintillation counter.

3. RESULTS AND DISCUSSION

At the holding voltage of −70 mV, the external application of an effective concentration of ATP (300 μM) in guinea pig brain RNA-injected oocytes induced an oscillatory inward current accompanied by an increase in membrane conductance (Fig. 1A: left panel). After a latency period of 28 ± 7 s (n = 8), this response peaked between 2 and 4 min after ATP application and decreased over the next 4–8 min. This current presented a reversal potential of −25 ± 4 mM (n = 5) and was completely inhibited by 9-anthracene carboxilic acid (9AC; final concentration = 2.5 mM; n = 5; data not illustrated), a blocker of chloride conductance [7]. These results demonstrate that these oscillations were generated by an inward chloride current. Native oocytes failed to respond to extracellular ATP even when perfused with concentrations up to 300 μM (Fig. 1A: right panel; n = 10). Intracellular injection of ATP did not cause any change in the current recording of injected oocytes. In view of these observations, it may be advanced that ATP effects, following mRNA injection, are due to newly expressed purinoceptors in oocyte membrane. To our knowledge, the ATP response we describe is the first demonstration of an expression of purinoceptors in *Xenopus* oocytes after injection of mRNA from mammalian brain. As depicted in Fig. 1B, the fluctuating current is not dependent upon extracellular Ca^{2+} since no significant modification either of the wave form or of the amplitude of this current could be recorded in a Ca^{2+}-free medium (Ca^{2+} 0, EGTA 1 mM) in the presence of 300 μM ATP (right panel; n = 4). By contrast, intracellular injection of EGTA (final concentration = 0.1 mM) markedly attenuated the ATP response (Fig. 1C; n = 8), suggesting that the ATP-sensitive chloride current was mainly connected to intracellular CA^{2+} variations probably resulting of release from an intracellular Ca^{2+} pool.

With the purpose of providing evidence for the involvement of intracellular Ca^{2+} stores in the ATP response, oocytes were challenged using

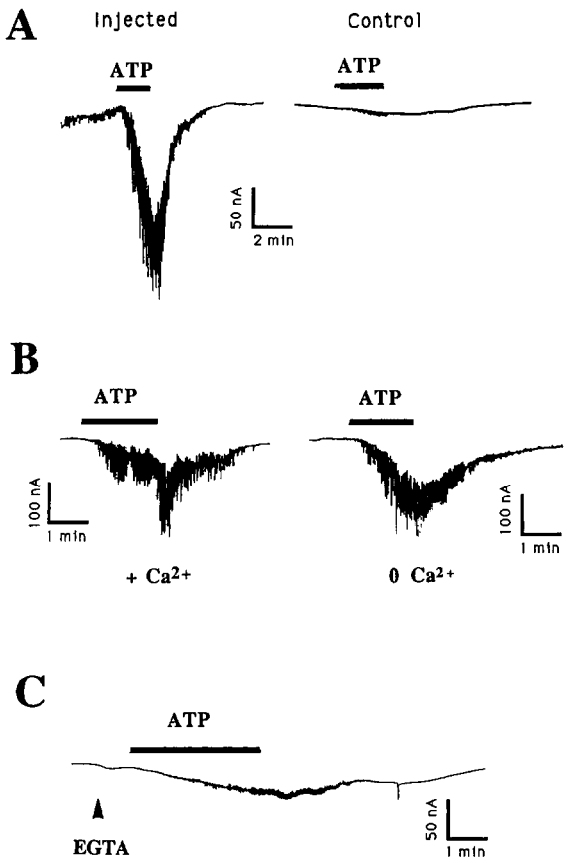

Fig. 1. ATP effects on membrane currents and Ca^{2+} dependence of the ATP response. (A) ATP-induced membrane currents compared in injected and control oocytes. (B) Dependence of ATP response upon external Ca^{2+} concentration: Ca^{2+} 1.8 mM (left panel); Ca^{2+} 0.1 mM EGTA (right panel). (C) Dependence of ATP response upon internal Ca^{2+} activity. Inhibition of ATP-evoked Cl-current by intraoocyte injection of EGTA (final concentration = 0.1 mM). 300 μM ATP superfusion are indicated by horizontal bars.

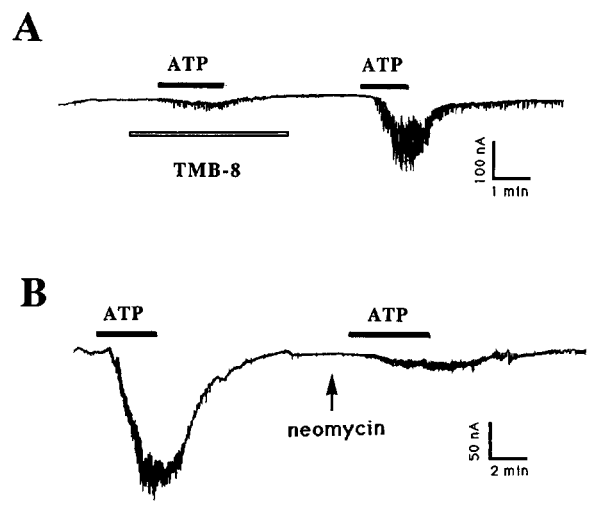

Fig. 2. Role of Ca^{2+} release from intracellular stores and mediation of ATP effects by phospholipase C. (A) Reversible inhibition by TMB-8 (external concentration of 100 μM) of the ATP-induced Cl-current. (B) Effect of intra-oocyte injection of 400 pmol neomycin on a Ca^{2+}-activated Cl^- current evoked during ATP superfusion. Oocytes were superfused with 300 μM ATP; TMB-8 superfusion is indicated by the empty horizontal bar.

3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester hydrochloride (TMB-8), a putative inhibitor of Ca^{2+} release from intracellular stores [12]. As shown in Fig. 2A, external application of this compound (final concentration = 100 μM) reversibly inhibited the ATP response (n = 5). One of the metabolic transduction pathways which could account for the purinergic response in injected oocytes is phosphoinositide breakdown induced by phospholipase C. Previous intracellular loading of oocytes with neomycin (400 pmol; Fig. 2B), an inhibitor of the enzymatic degradation of PIP_2 and PIP [13], strongly reduced the purinergic response (n = 5). These experiments suggest that phospholipase C activation, leading to Ins(1,4,5)P_3 synthesis, underlies the ATP-induced oscillatory chloride current. Ins(1,4,5)P_3 production following purinoceptor activation was investigated by measuring the level of this water-soluble product in cells prelabelled with the precursor myo-[2-^{3}H]inositol. As shown in Fig. 3A, a significant increase of Ins(1,4,5)P_3 could be detected 2 min after bathing oocytes with 300 μM ATP (301 ± 45% of control; 3 experiments). This stimulation of Ins(1,4,5)P_3 production with ATP was comparable with the ACh-induced rise of Ins(1,4,5)P_3 formation in injected oocytes (391 ± 52% of control; 3 experiments). The Ins(1,4,5)P_3 response of this Ca^{2+} mobilizing neurotransmitter may be considered as a reference positive control since the muscarinic receptor transduction pathway has been established in previous studies as a Ca^{2+} release from Ins(1,4,5)P_3-sensitive Ca^{2+} stores in defolliculated oocytes injected with mammalian brain mRNA [14]. Moreover, Ins(1,4,5)P_3 level remained unchanged in native oocytes in the presence of ATP (300 μM; data not shown; 3 experiments). To release Ca^{2+}, Ins(1,4,5)P_3 must bind to receptors that are somehow linked to Ca^{2+} channels connected with Ins(1,4,5)P_3-sensitive pools. Heparin inhibits both the binding of Ins(1,4,5)P_3 to its purified receptor [15] and the mobilization of Ca^{2+} in numerous cell systems [16]. Fig 3B shows that intra-oocyte injection of heparin (final concentration = 80 μM) blocked the ATP responsiveness (n = 4). Therefore, Ins(1,4,5)P_3-induced Ca^{2+} mobilization did appear to be involved in the ATP effects. Previous pharmacological studies (see [17] for review), based on the rank order of potency of various purinergic agonists, have led to establishing a classification of purinergic receptors as either P_1 (recognizing adenosine and AMP) or P_2 (recognizing ADP and ATP). As shown in Fig. 4, ATP and ADP (final concentration = 300 μM) were quipotent in eliciting the fluctuating chloride current (n = 5) while AMP and adenosine (same concentration) were ineffective in guinea pig brain RNA-injected oocytes (n = 5). Therefore, the acquired purinoceptor may be made apparent to a P_2 receptor according to Burnstock's classification.

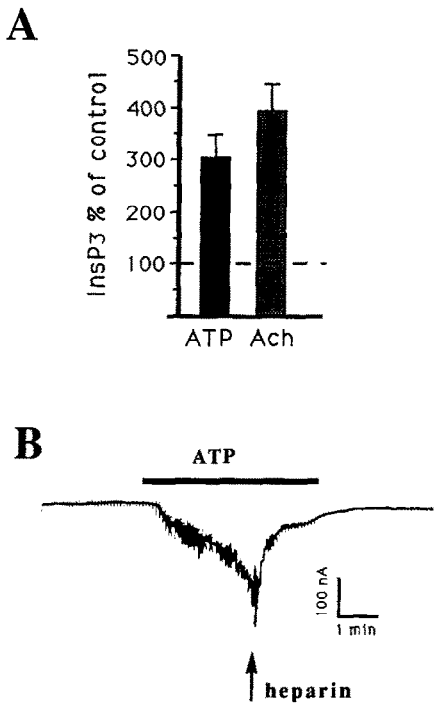

Fig. 3. Mediation of the ATP response by Ins(1,4,5)P_3. (A) Accumulation of InsP_3 in injected oocytes followed for 2 minutes in the presence of 300 μM ATP or 10 μM ACh. Results are expressed as the percentage of basal level. (InsP_3 % of control) means ± SE, three experiments (duplicates); 100% represents the amount of InsP_3 in the absence of external ATP. (B) Typical effect of intraoocyte injection of heparin (final concentration = 80 μM) on Ca^{2+} activated fluctuating current evoked during the ATP superfusion. The oocyte was superfused with 300 μM ATP.

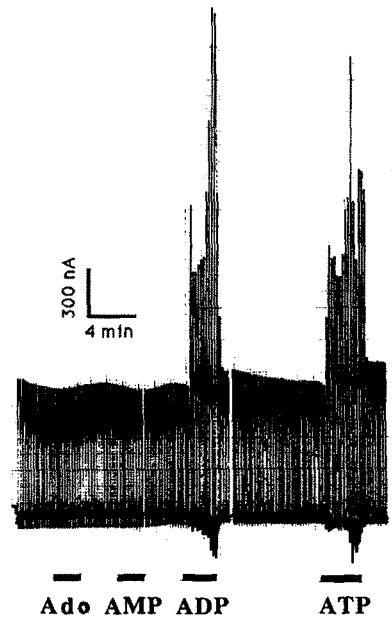

Fig. 4. Membrane currents induced by purinergic agonists in oocytes. Typical chart record of the effects of adenosine (Ado), AMP, ADP, ATP. Each compound was superfused at the concentration of 300 μM. The oocyte was repeatedly depolarized to 20 mV for 4 s every 20 s to test the membrane conductance variations evoked by the different purinergic agonists.

Our results bring several lines of evidence for a stimulation by ATP of phosphoinositide breakdown evoked by activation of newly expressed purinoceptors. They are consistent with previous studies which have demonstrated that P_2 receptor occupation increases phosphatidyl inositol polyphosphate turnover with subsequent production of Ins(1,4,5)P_3 in a wide variety of cell systems including isolated hepatocytes, Ehrlich ascite tumor cells and rat ventricular cells [18–20]. Likewise, recent findings demonstrated that extracellular ATP caused a large increase in inositol phosphate accumulation in neural cells [21] and thereby initiate a chain of events, which may play a role in the induction of long-term adaptative changes (long-term potentiation; LTP) in synaptic neuronal function. The biochemical characterization of the P_2 class receptors is hardly advanced; no nucleic or immunological probes are available, thus preventing the use of classical techniques to study purinoceptors. Our results suggest an alternative way to investigate a functional and structural characterization of this type of receptor. This approach would be to use the *Xenopus* oocyte to clone the receptor gene, as was previously done with the 5-HT_{1C} or the substance K receptors [22,23].

Acknowledgements: This work received financial support from the Association Française contre les Myopathies.

REFERENCES

[1] Holton, F.A. and Holton, P. (1954) J. Physiol. (Lond.) 126, 124–140.
[2] Burnstock, G. (1981) J. Physiol. (Lond.) 313, 1–35.
[3] Jahr, C.E. and Jessel, T.M. (1983) Nature 304, 730–733.
[4] Fyffe, R.E.W. and Perl, E.R. (1984) Proc. Natl. Acad. Sci. USA 81, 6890–6893.
[5] Richardson, P.J. and Brown, S.J. (1987) J. Neurochem. 48, 622–630.
[6] Burnstock, G. and Kennedy, C. (1985) Gen. Pharmacol. 16, 433–440.
[7] Dascal, N. (1987) CRC Crit. Rev. Biochem. 22, 317–387.
[8] Miledi, R. (1982) Proc. R. Soc. Lond. B 215, 491–497.
[9] Chirgwin, J.M., Przybyla, A.E., Mac Donald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[10] Fournier, F., Honoré, E., Brulé, G., Mironneau, J. and Guilbault, P. (1989) Plüggers Arch. 413, 682–684.
[11] Berridge, M.J., Downes, C.P. and Hanley, H.R. (1982) Biochem. J. 206, 595–597.
[12] Chiou, C.Y. and Malagodi, M.H. (1975) Br. J. Pharmacol. 53, 279–285.
[13] Cockcroft, S. and Gomperts, B. (1985) Nature 314, 534–536.
[14] Nomura, Y., Kaneko, S., Kato, K., Yamagishi, S. and Sugiyama, H. (1987) Mol. Brain Res. 2, 113–123.
[15] Supattapone, S., Worley, P., Baraban, J. and Snyder, S. (1988) J. Biol. Chem. 263, 1530–1534.
[16] Worley, P.F., Baraban, J.M., Supattapone, S., Wilson, V.S. and Snyder, S.H. (1987) J. Biol. Chem. 262, 12132–12136.
[17] Gordon, J.L. (1986) Biochem. J. 233, 309–319.
[18] Charest, R., Blackmore, P.F. and Exton, J.H. (1985) J. Biol. Chem. 260, 15789–15794.
[19] Dubyak, G.R. and De Young, M.B. (1985) J. Biol. Chem. 260, 10653–10661.
[20] Legssyer, A., Poggioli, J., Renard, D. and Vassort, G. (1988) J. Physiol. (Lond.) 401, 185–199.
[21] Ehrlich, Y.H., Snider, R.M., Kornecki, E., Garfield, M.G. and Lenox, R.H. (1988) J. Neurochem. 50, 295–301.
[22] Julius, D., MacDermott, A.B., Axel, R. and Jessel, T.M. (1988) Science 241, 558–564.
[23] Masu, Y., Nakayama, K., Tamaki, H., Harada, Y., Kuno, M. and Nakanishi, S. (1987) Nature 329, 836–838.