Potential of miniature endplate potential frequency by ATP in *Xenopus* tadpoles

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1 Extracellular application of ATP (1 mM), a substance co-stored and co-released with acetylcholine in peripheral nervous systems, potentiated the spontaneous secretion of acetylcholine (ACh) but had no effect on the amplitude and decay time constant of miniature endplate potentials (m.e.p.ps) at neuromuscular synapses in *Xenopus* tadpoles.

2 α,β -Methylene ATP (0.3 mM) and GTP (1 mM) were also effective in increasing m.e.p.p. frequency. On the other hand, ADP, AMP and adenosine (all at 1 mM) decreased m.e.p.p. frequency.

3 Unlike the transient effect of ATP analogue and GTP on m.e.p.p. frequency, the phorbol ester TPA (2 μ M) which is a protein kinase C activator, increased m.e.p.p. frequency consistently and the effects lasted as long as the presence of TPA.

4 Staurosporine (0.5 μ M) and H-7 (10 μ M), which are protein kinase C inhibitors, each decreased the basal level of m.e.p.p. frequency and markedly inhibited the effects of both ATP and TPA.

5 These results suggest that there is a basal activity of cytosolic protein kinases in the nerve terminals of *Xenopus* tadpoles and the effect of ATP is probably mediated by the binding of membrane surface purinoceptors which in turn activates cytosolic protein kinases and increases ACh release.

Keywords: ATP; protein kinase activation; ACh release

Introduction

Cell-cell interactions during the early phase of synaptogenesis are of crucial importance to the formation of neuronal connections. These interactions are responsible for cell-cell recognition and selective adhesion, and initiate the structural and functional differentiation in pre- and postsynaptic cells. The vertebrate neuromuscular junction forms as a result of intimate interactions between two specialized cells. Development of synaptic connections depends on electrical and trophic interactions between pre- and postsynaptic cells (Purves, 1986; Frank, 1987; Shatz, 1990). Release of trophic factors from the presynaptic nerve terminals may regulate the distribution and the ion-conducting properties of transmitter receptors in the postsynaptic membrane (Usdin & Fishbach, 1986; Nitkin *et al.*, 1987; Laufer & Changeux, 1987; Mülle *et al.*, 1988). Factors secreted by the postsynaptic cell may regulate the growth and survival of presynaptic neurones (Henderson *et al.*, 1981; Gurney, 1984; Oppenheim & Haverkamp, 1988). It is now widely recognized that many mature neurones contain more than one neurotransmitter (Hokfelt *et al.*, 1984), and in at least some instances, both substances have physiological effects on the postsynaptic cell (see, for example, Jan *et al.*, 1983). Adenosine-5'-triphosphate (ATP) is known to be released in association with acetylcholine (ACh) at synapses in the vertebrate peripheral nervous system (Silinsky & Hubbard, 1973; Silinsky, 1975; Zimmerman, 1978). Muscle cells are also known to secrete substantial amounts of ATP in response to electrical activity (Smith & Lindgren, 1986). Exogenous ATP and its derivative adenosine, at mature synapses, have inhibitory effects on the evoked and spontaneous ACh release (Ribeiro & Walker, 1975; Silinsky & Ginsborg, 1983), suggesting that ATP may serve as a negative feedback signal for regulating transmitter release. We were the first to report that ATP greatly potentiates the spontaneous ACh release at developing neuromuscular synapses in *Xenopus* cell culture (Fu & Poo, 1991). Since spontaneous ACh release is known to regulate the

development of contractile properties of the postsynaptic muscle cell (Kidokoro & Saito, 1988), extracellular ATP may serve as a positive trophic factor at developing neuromuscular synapses. We report here that extracellular ATP also causes an increase in the frequency of miniature endplate potentials (m.e.p.ps) of *Xenopus* tadpoles, although the effects are much smaller than that of cultured cells. However, it is interesting to find that there are developmental changes in the modulation of ACh release by ATP.

Methods

The experiments were performed on the myotomal cells of tadpoles of *Xenopus laevis*. Stages of development of tadpoles of *Xenopus* were assessed by the criteria of Nieuwkoop & Faber (1967). We used tadpoles from stages 49 to 52 (about 2 to 3 weeks-old embryos). Tadpoles were placed in Ringer solution (composition mM: NaCl 115, CaCl₂ 2, KCl 2.5, HEPES 10, pH 7.3), where skin, head and viscera were gently removed, leaving the skinned tail. The tail then was transferred to a small sylgard-lined petri dish and pinned out on its side, and 1–3 μ M tetrodotoxin and 10 mM CaCl₂ were added to the bathing solution to avoid muscle contraction and improve membrane resealing, respectively.

Synaptic miniature endplate potentials (m.e.p.ps) were measured by standard microelectrode techniques. The microelectrodes were filled with 3 M potassium acetate and had resistances ranging from 5–10 M Ω . All experiments were performed at room temperature (23–25°C). M.e.p.ps were processed on a digital waveform analyzer (Data 6100)-computer system. To avoid the large variance of the m.e.p.p. frequency between different endplates, we used the same endplate to monitor the effects of drugs. Spontaneous quantal release was estimated as the ratio of frequency after and before drug treatment. The results are expressed as the mean $\pm$ s.e.mean. The statistical significance was evaluated by Student's *t* test. However, ANOVA and Kruskal-Wallis one-way analysis were used to evaluate the statistical significance between the histograms in Figure 3.

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The following chemicals were used: ATP, ADP, AMP, adenosine, GTP, α,β -methylene ATP, TPA (phorbol 12-myristate 13-acetate) (+)-tubocurarine and tetrodotoxin (Sigma); staurosporine (Boehringer) and H-7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride) (Seikagaku Kogyo Co., Japan).

Results

Effects of ATP on the miniature endplate potentials (m.e.p.ps)

In the tadpoles of stages 49 to 52 of *Xenopus*, the resting membrane potential was -80 to -100 mV. The mean frequency of m.e.p.p. was $118.6 \pm 18.3 \text{ min}^{-1}$ ($n = 42$). Many of these spontaneous ACh releases are capable of eliciting action potentials and contractions in the muscle cells. From observation under the microscope, application of $7 \mu\text{M}$ (+)-tubocurarine completely abolished the spontaneous contractions of the tadpoles. Therefore, m.e.p.ps were recorded from the same endplate in the presence of $1-3 \mu\text{M}$ tetrodotoxin to prevent muscle contractions. Figure 1a showed the effect of bath-applied 1 mM ATP on the frequency of m.e.p.ps. Each curve connects data collected from 1 synapse. The m.e.p.p. frequency increased transiently soon after ATP application, and the potentiation gradually subsided within 5 min. Figure 2 shows representative oscilloscopic traces of m.e.p.ps before (Figure 2a) and after (Figure 2b) ATP treatment. In addition, we examined the effects of α,β -methylene ATP, a slowly degraded derivative of ATP. We found that application of 0.3 mM α,β -methylene ATP also increased m.e.p.p. frequency transiently (Figure 1b). The increase of m.e.p.p. frequency by ATP is unlikely to result from the postsynaptic action of ATP because the m.e.p.p. amplitude histogram was unaffected by ATP (Figure 3). The average m.e.p.p. amplitude and

Figure 2 Potentiation of spontaneous acetylcholine (ACh) release by adenosine 5'-triphosphate (ATP). Samples of miniature endplate potentials (m.e.p.ps) before (a) and after (b) 1 mM ATP application are shown on the oscilloscope records. Scale bars are 2 mV and 20 ms , respectively.

Figure 1 The effect of adenosine-5'-triphosphate (ATP) analogues on the frequency of miniature endplate potentials (m.e.p.ps). ATP at 1 mM (a) or α,β -methylene ATP at 0.3 mM (b) was bath applied at the time marked by the arrow. Each curve connects data collected from 1 synapse. Note that both drugs increased m.e.p.p. frequency transiently.

Figure 3 The effect of adenosine-5'-triphosphate (ATP) on the amplitude distribution of miniature endplate potentials (m.e.p.ps). Compared with control (a), 1 mM ATP had no significant effect on the m.e.p.p. amplitude (b). Amplitude histograms constructed from 140 m.e.p.ps.

decay time constant before and after ATP treatment was 1.56 ± 0.16 mV, 3.52 ± 0.13 ms and 1.34 ± 0.12 mV, 3.68 ± 0.13 ms, respectively ($n = 9$).

Potency of related nucleotides

Previous studies have indicated that P_2 -purinoceptors mediate extracellular ATP effects in many cell types. We have examined the effects of various nucleotides on m.e.p.s. We found that adenosine diphosphate (ADP), adenosine monophosphate (AMP) or adenosine (all at 1 mM) did not increase m.e.p.p. frequency but instead, decreased it (Figure 4). The ratios of the minimal m.e.p.p. frequency after treatment of various nucleotides to the control frequency were $61.6 \pm 2.5\%$ ($n = 6$), $48.2 \pm 6.0\%$ ($n = 9$) and $30.5 \pm 7.1\%$ ($n = 5$) for ADP, AMP and adenosine, respectively. These results indicated that the enhancing effect of ATP on ACh secretion was due to the action of ATP itself, not to any of its hydrolytic products. To our surprise, guanosine triphosphate (GTP) at 1 mM also increased m.e.p.p. frequency transiently as ATP did (Figure 5). The ratio of the maximal m.e.p.p. frequency after treatment of GTP to the control frequency was $295 \pm 55\%$ ($n = 5$). These results were summarized in Figure 6; ATP, α,β -methylene ATP and GTP increased but ADP, AMP and adenosine decreased m.e.p.p. frequency of tadpoles of *Xenopus*.

Involvement of protein kinases

It has been previously reported that ATP application to myotube cultures triggers the formation of inositol trisphosphate and diacylglycerol, which are known to activate protein kinase C (PKC). Enhancement of PKC activity by direct

Figure 4 Effects of ADP, AMP and adenosine on the frequency of miniature endplate potentials (m.e.p.s). Bath application of ADP (a), AMP (b) or adenosine (c) (at 1 mM each) at the time marked by the arrow decreased the frequency of m.e.p.s. Each curve represents the data collected from 1 synapse.

Figure 5 Effect of guanosine-5'-triphosphate (GTP) on the frequency of miniature endplate potentials (m.e.p.s). Bath application of 1 mM GTP at the time marked by the arrow transiently increased the frequency of m.e.p.s. Each curve represents the data collected from 1 synapse.

activation with phorbol esters is also known to increase transmitter release. To determine whether ATP potentiates the spontaneous ACh release by activating PKC, we have examined the effect of kinase inhibitors. Figure 7 showed that pretreatment with staurosporine (at $0.5 \mu\text{M}$), a PKC inhibitor (Tamaoki *et al.*, 1986), completely inhibited the potentiating effect of ATP. As shown in Figure 7, pretreatment with staurosporine by itself caused a marked inhibition of the basal level of spontaneous ACh release. The ratio of minimal m.e.p.p. frequency after treatment with staurosporine to the control frequency was $36.3 \pm 4.2\%$ ($n = 7$). Another protein kinase C inhibitor, H-7 ($10 \mu\text{M}$) also had similar effects, inhibiting the basal level of m.e.p.p. and ATP effects by $40.0 \pm 5.9\%$ and $66.3 \pm 13.5\%$, respectively ($n = 4$). For comparison, we also examined the effects of phorbol ester, TPA. TPA at $2 \mu\text{M}$ increased m.e.p.p. frequency for a long period as shown in Figure 8a. Pretreatment with staurosporine ($0.5 \mu\text{M}$) also significantly reduced the potentiation effect of TPA (Figure 8b). These are two similar replicates of these experiments. Taken together, these results are consistent with the notion that the ATP effect is probably mediated by PKC and that there is a tonic PKC activity in motor nerve terminals of tadpoles.

Figure 6 Effects of purine nucleotides on the frequency of miniature endplate potentials (m.e.p.s). The maximum effects of purine nucleotides on m.e.p.p. frequency were determined. Note that ATP, α,β -methylene ATP and GTP increased but ADP, AMP and adenosine decreased m.e.p.p. frequency (all at 1 mM except α,β -methylene ATP which is 0.3 mM). Data are presented as mean $\pm$ s.e. (vertical bars) ($n = 4-9$). * $P < 0.05$ as compared with control.

Figure 7 Antagonism by staurosporine of the potentiation effect of ATP on the frequency of miniature endplate potentials (m.e.p.ps). Pretreatment with staurosporine ($0.5 \mu\text{M}$) alone inhibited the m.e.p.p. frequency markedly. ATP was applied 15 min later and the potentiation effect was completely abolished by staurosporine. Each curve connects the data collected from 1 synapse.

Figure 8 Effects of TPA (phorbol 12-myristate 13-acetate) on the frequency of miniature endplate potentials (m.e.p.ps). TPA ($2 \mu\text{M}$) was added at the time marked by the arrow and m.e.p.p. frequency was increased consistently and lasted as long as the presence of TPA (a). Pretreatment with staurosporine ($0.5 \mu\text{M}$) inhibited the potentiating action of TPA on m.e.p.p. frequency (b). Each curve connects the data obtained from 1 synapse.

Discussion

ATP is co-stored with ACh in the vesicle fraction of nerve endings (Dowdall *et al.*, 1974) and is a potential trophic substance at peripheral synapses. Physiological stimulation of a number of neuromuscular preparations has been shown to result in ATP release into the extracellular medium (Boyd & Forrester, 1968; Silinsky & Hubbard, 1973; Meunier *et al.*,

1975). However, ATP may also be released from stimulated postsynaptic cells. This has been observed in crayfish (Smith & Lindgren, 1986), frog (Abood *et al.*, 1962) and human muscle (Forrester, 1972) as well as in *Torpedo* electric organ (Israel *et al.*, 1976). Moreover, exogenous ATP has been shown to reduce transmitter release from presynaptic terminals of preganglionic sympathetic neurones (Silinsky & Ginsborg, 1983) and motoneurons (Ribeiro & Walker, 1975). We first found that ATP suppresses evoked ACh release, but greatly potentiates spontaneous ACh release in *Xenopus* cell culture (Fu & Poo, 1991). In the present study, we further demonstrated the positive regulation of ACh release by ATP in *Xenopus* tadpoles and suggest a possible positive trophic action of ATP during embryonic development.

That ATP had no effect on the m.e.p.p. amplitude distribution histogram further supported the idea that the increase in m.e.p.p. frequency resulted from the presynaptic increase of ACh release by ATP. The potentiation of ACh release by ATP could result directly from the action of ATP itself or of its hydrolytic products. Since we found that ADP, AMP and adenosine inhibited the frequency of m.e.p.ps, ATP must potentiate ACh release by itself, presumably through binding with cell surface ATP receptors. Although ADP is not as effective an agonist as ATP, GTP had a profound potentiating effect on the frequency of m.e.p.ps. GTP has been reported to act on P_2 -purinoceptors in many cell systems (Keppens *et al.*, 1989; Okajima *et al.*, 1989). Taken together with the potentiating action of α, β -methylene ATP, ATP may act on cell surface P_2 -purinoceptors. The action of GTP in tadpoles is quite different from its effects on *Xenopus* cell culture (plated from Day-1 embryos of stages 22-23) in which GTP produced no effect on spontaneous ACh release (Fu & Poo, 1991). On the other hand, adenosine produces no inhibition of ACh release in *Xenopus* cell culture (Fu & Poo, 1991) but inhibited ACh release in *Xenopus* tadpoles, which is very similar to the action at the mature nerve terminals and where it is supposed to act on adenosine A_1 receptors (Moody & Burnstock, 1982; Schubert *et al.*, 1982; Stone, 1983; Dunwiddie, 1984). The inhibitory action of ADP and AMP on m.e.p.p. frequency probably results from the hydrolytic product of adenosine as reported before (De May *et al.*, 1979; Silinsky, 1980). It is very interesting to find that compared with cell culture of earlier embryonic stages (stages 22-23), the potency of ATP is decreased (in *Xenopus* cell culture 0.3 mM ATP increased m.e.p.c. frequency a few hundred fold) and the effects of adenosine appeared in *Xenopus* tadpoles, which represent the later stages of embryos (stages 49 to 52). In recent years, increasing attention has been focused on age-related changes in receptor function. For instance, there are also developmental changes in the β -adrenoceptor modulation of L-type calcium current in enzymatically isolated adult and newborn rabbit ventricular cells (Osaka & Joyner, 1992). Thus, the potentiating action of spontaneous ACh release by ATP at earlier embryonic stage may be related to its positive trophic action.

Many hormones and neurotransmitters exert their calcium-mobilizing effects through G protein-mediated activation of phospholipase C (PLC), which hydrolyzes phosphatidylinositol biphosphate (PIP_2). This generates two second messengers, inositol 1,4,5-trisphosphate (IP_3), involved in the release of calcium from internal stores, and diacylglycerol (DAG) which activates protein kinase C (PKC) (Berridge, 1987). The phosphorylation product of IP_3 , inositol 1,3,4,5-tetrakisphosphate (IP_4), has been proposed as an intracellular second messenger regulating Ca^{2+} influx from the extracellular to the intracellular space (Irvine & Moor, 1986; Morris *et al.*, 1987). The responses of several cell types to extracellular ATP involve phosphoinositide (PI) metabolism, ATP acting on P_2 -purinoceptors coupled via G proteins to PLC (Hagglad & Heilbronn, 1987; Cockcroft & Stutchfield, 1989; Heilbronn & Eriksson, 1989; El-Moatassim *et al.*, 1992). Enhancement of PKC activity by direct activation with phorbol esters is also known to increase transmitter release (Shapira *et al.*,

1987). Since addition of staurosporine and H-7 inhibited the ATP effect, the action of ATP on the nerve terminals is probably mediated by PKC. Because of the lack of specificity of these inhibitors, involvement of other kinases cannot be excluded. In the absence of ATP, staurosporine as well as H-7 by itself inhibited significantly the basal level of spontaneous ACh release, suggesting that there is a tonic kinase activity in *Xenopus* tadpoles. Compared with the transient effect of ATP, TPA caused a prolonged increase in the frequency of m.e.p.s, indicating that the cascades of transmitter release behind the purinoceptor activation behave normally. Thus, the decline in the ACh release frequency could be due to the desensitization of ATP purinoceptors. A similar desensitizing effect of ATP has been also observed in other cell systems (Jahar & Jessell, 1983; Krishtal *et al.*, 1983; Hume & Honig, 1986).

Spontaneous ACh release at the developing neuromuscular synapse may play a trophic function in the synapse maturation.

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