ELSEVIER

European Journal of Pharmacology 282 (1995) 213–217

ejp

Possible neuronal origin of ATP release evoked by forskolin and ouabain from guinea-pig atrial segments

Takeshi Katsuragi [a,*], Takeo Tokunaga [a], Chiemi Sato [b], Tatsuo Furukawa [a]

[a] *Department of Pharmacology, School of Medicine, Fukuoka University, Fukuoka 814-80, Japan*
[b] *Research Laboratory of Biodynamics, School of Medicine, Fukuoka University, Fukuoka 814-80, Japan*

Received 2 February 1995; revised 29 May 1995; accepted 2 June 1995

Abstract

The characteristics of ATP release evoked by forskolin and ouabain from atrial segments of guinea-pig were evaluated under electrical stimulation. Forskolin (1 μM) produced a massive release of ATP together with a positive inotropic response. Both 30 μM W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide · HCl), a calmodulin antagonist, and 30 μM vinblastine, a mitotic inhibitor, markedly inhibited the evoked release of ATP without affecting the evoked contraction. However, 100 μM *N*-ethylmaleimide abolished completely the basal and drug-evoked ATP release and further the evoked contraction. Both the ATP release and contraction evoked by ouabain (3 μM) were similarly affected by W-7, vinblastine and *n*-ethylmaleimide. The release of ATP, but not the contraction, evoked by forskolin was strongly suppressed by 10 μM okadaic acid, a protein phosphatase inhibitor. The suppression by okadaic acid of the evoked release was thoroughly antagonized in the presence of 0.01 μM PMA (phorbol 12-myristate 13-acetate), but not 10 μM H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine). These results suggest that forskolin, like ouabain, may dominantly cause the neuronal release of ATP from cardiac adrenergic nerves, although the possible participation of release from muscular sources cannot be ignored.

Keywords: ATP release, neuronal; Forskolin; Ouabain; Atrium, guinea-pig; W-7; Vinblastine; Okadaic acid

1. Introduction

Since the pioneering work by Su et al. (1971), ATP has been considered to be a potential co-transmitter which is released together with classical transmitters from the autonomic nervous system (Su, 1983; Katsuragi and Furukawa, 1985; Burnstock, 1986; Von Kügelgen and Starke, 1991). Especially, evidence has accumulated that ATP can be released by electrical or chemical stimulation from adrenergic nerves of the mammalian vas deferens (Sneddon et al., 1982; Kirkpatrik and Burnstock, 1987; Katsuragi et al., 1988) and vascular tissues (Katsuragi and Su, 1980; Sneddon and Burnstock, 1985; Von Kügelgen and Starke, 1985). However, evidence for ATP release from the neurons of mammalian heart is still sparse. In recent studies, we reported that isoprenaline and forskolin, cyclic AMP increasers, stimulated the release of ATP and had a positive inotropic action in atrial, but not auricle, segments of guinea-pig under electrical stimulation (Katsuragi et al., 1993b). Ouabain, a cardiac glycoside, is known to facilitate the release of noradrenaline from adrenergic neurons of rat heart (Kranzhöfer et al., 1991) and guinea-pig vas deferens (Katsuragi et al., 1988, 1994). In addition to noradrenaline release, we have shown the possibility that ATP is released from pulmonary artery segments of rabbit by adding ouabain (Katsuragi and Su, 1982), predicting that the glycoside would elicit the release of ATP from cardiac segments.

Accordingly, in the present study, we attempted to clarify the characteristics of ATP release from guinea-pig atrial segments evoked by forskolin and ouabain in the presence of a calmodulin antagonist (W-7), a mitotic inhibitor (vinblastine), a protein phosphatase inhibitor (okadaic acid), etc.

* Corresponding author. Tel. 092-801-1011, fax 092-865-4384.

SSDI 0014-2999(95)00341-X

2. Materials and methods

2.1. Preparation of atrial segment

Male guinea-pigs (250–350 g) were stunned and bled. The heart was quickly dissected and placed in a dish filled with oxygenated (95% O_2 plus 5% CO_2) Krebs' solution (37°C). After the left atrium was immediately isolated from the heart, blood and clots around the tissue were removed. The left atrium (size, approximately 4 mm × 8 mm; wet weight, 0.0157 ± 0.0011 g, $n = 9$) was suspended in a bath (1 ml) filled with Krebs' solution (37°C) of the following composition (mM): NaCl, 122; KCl 5.2; $CaCl_2 \cdot 2H_2O$, 2.4; $MgSO_4 \cdot 7H_2O$, 1.2; $NaHCO_3$, 5.6; D-glucose, 11.0; ascorbic acid, 0.1 and Na_2EDTA, 0.03. The bathing medium was constantly bubbled through a flow regulator with 95% O_2 + 5% CO_2.

After being loaded with 0.5 g resting tension, the preparation was placed between a pair of platinum electrodes, and then electrical stimulation (3 ms, 4 Hz, supramaximal voltage) was continuously applied to the segment through platinum electrodes. Electrically evoked contractions of the left atrium were monitored on a polygraph with a force transducer (Toyo-Baldwin, T-7-8-240).

The concentration of organic solvents such as ethanol was limited to 0.1% of the bathing medium without any effect.

2.2. Measurement of ATP release

The preparation, under electrical stimulation, was allowed to equilibrate for 40 min in Krebs' solution and, during the equilibration, this medium was replaced once with fresh Krebs' solution (1 ml, 37°C). A sample medium (50 μl) as control was taken from the bathing medium 5 min before administration of drugs. Subsequently, test samples of 50 μl were collected 5, 10 and 15 min after administration of drugs. After every sampling, an equal volume of bubbled Krebs' solution (37°C) was added to the bath. At the end of the experiment, the preparation was blotted with a filter paper and weighed. In order to determine endogenous ATP, each sample of medium was allowed to react with 100 μl of ATP reagent solution (Lucifel-LU, Kikkoman, Noda, Japan). The intensity of light produced by the reaction was measured by a luminometer (NU-600, Niti-on, Funabashi, Japan) as described previously (Katsuragi et al., 1988, 1991).

2.3. Drugs

The drugs used were ouabain (E. Merk, Darmstadt, Germany); forskolin, vinblastine sulfate, PMA (phorbol 12-myristate 13-acetate) (Sigma, St. Louis, MO, USA); W-7 (*N*-(6-aminohexyl)-1-naphthalene sulfonamide), H-7 (1-(5-isoquinolinesulfonyl)-2-methyl piperazine) (Seikagaku Co., Nagoya, Japan); NEM (*N*-ethylmaleimide) (Nacalai, Kyoto, Japan) and okadaic acid (Wako, Osaka, Japan).

2.4. Statistical analysis

Differences between multiple means were tested for statistical significance by one-way analysis of variance (ANOVA) followed by Dunnett's test. A $P < 0.05$ was considered to be significant.

3. Results

3.1. ATP release evoked by forskolin and ouabain

Samples of medium from baths containing segments of guinea-pig left atrium under electrical stimulation were collected 5, 10 and 15 min after administration of the test cardiotonics. The release of ATP was maximal 5 min after the addition of forskolin or ouabain and then declined gradually up to 15 min (Fig. 1).

The amount of ATP released at these times points after exposure to 1 μM forskolin and 3 μM ouabain was 83.32 ± 11.66, 62.70 ± 4.84 and 51.13 ± 10.85 pmol/g wet weight ($n = 7$), and 57.35 ± 3.39, 54.26 ± 3.68 and 45.83 ± 4.22 pmol/g wet weight ($n = 8$), respectively. Similarly, the basal release of ATP without the cardiotonics was 35.16 ± 0.72, 27.72 ± 1.44 and 26.37 ± 1.79 pmol/g wet weight ($n = 5$), respectively.

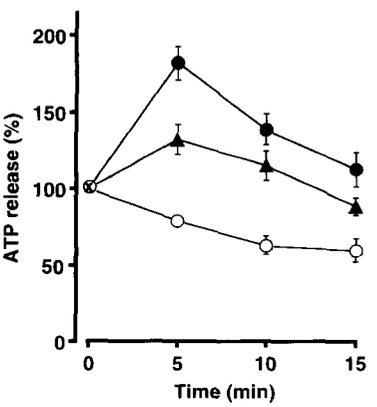

Fig. 1. Time course of ATP release evoked by forskolin and ouabain from left atrial segments of guinea-pig. (○) basal ATP release ($n = 5$); (●) ATP release evoked by 1 μM forskolin (n = 7); (▲) ATP release evoked by 3 μM ouabain ($n = 8$). Values are expressed as percentage (mean ± S.E.M.) of control (100%) before administration of cardiotonics. Net mean ATP release (evoked release − basal release) 5 min after exposure to forskolin and ouabain was approximately 48.16 and 22.19 pmol/g wet weight, respectively.

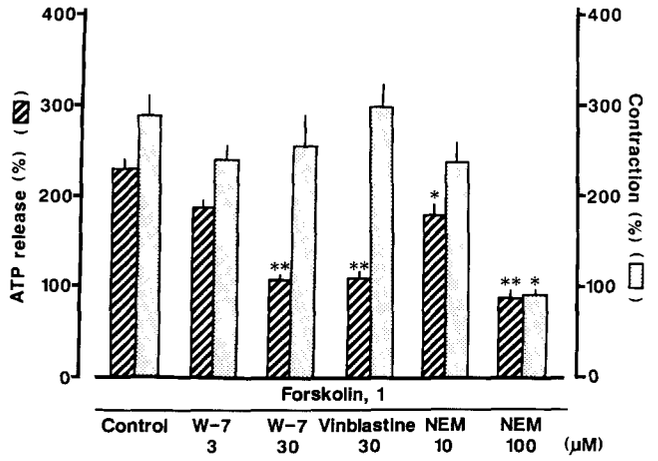

Fig. 2. Effects of W-7, vinblastine and *n*-ethylmaleimide on ATP release and contraction evoked by forskolin. Values for ATP release (hatched columns) and contraction (grey columns) are the maximum responses expressed as percentage (mean ± S.E.M.) of basal control before forskolin (100%) from 4–6 experiments. The numbers mean the concentrations of the drugs. Test inhibitors were introduced to the bath 30 min before forskolin. $^{*}P < 0.05$, $^{**}P < 0.01$ from corresponding control.

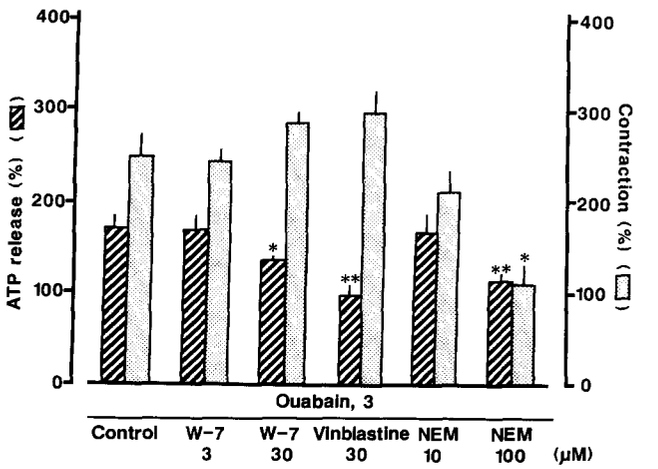

Fig. 3. Effects of W-7, vinblastine and *n*-ethylmaleimide on ATP release and contraction evoked by ouabain. Values for ATP release (hatched columns) and contraction (grey columns) are the maximum responses expressed as percentage (mean ± S.E.M.) of basal control before ouabain (100%) from 4–8 experiments. The numbers mean the concentrations of the drugs. Test inhibitors were added to the bath 30 min before ouabain. $^{*}P < 0.05$, $^{**}P < 0.01$ from corresponding control.

3.2. Effects of W-7, vinblastine and NEM on ATP release and contraction evoked by forskolin and ouabain

The release of ATP evoked by 1 μM forskolin and 3 μM ouabain was coupled with a facilitation of the contraction of atrial segments. The maximum contractions induced by forskolin and ouabain appeared immediately and within 3 min of drug administration, respectively. The relationships between the maximum values of ATP release and contraction evoked by the cardiotonics were examined in the presence and absence of inhibitors such as W-7. The release of ATP elicited 5 min after exposure to either forskolin or ouabain was markedly reduced by administration of W-7 and vinblastine at the concentrations of 30 μM. Nevertheless, the evoked contraction was virtually unaffected by these procedures. The effect of *n*-ethylmaleimide at 10 μM on the contraction tended to show the same pattern as that for W-7 (30 μM) and vinblastine. However, 100 μM *n*-ethylmaleimide prevented completely not only the release of ATP but also the contractile response.

These results are illustrated in Fig. 2 and Fig. 3.

The basal release (100%) of ATP was virtually uninfluenced by W-7 (115.3 ± 23.7%, $n = 7$) and vinblastine (115.0 ± 16.5%, $n = 5$) even at a concentration of 30 μM, but was halved by 100 μM *N*-ethylmaleimide (50.0 ± 11.7%, $n = 5$).

3.3. Effect of okadaic acid on ATP release evoked by forskolin

Basal ATP release and ATP release in the presence of 10 μM okadaic acid, a protein phosphatase in-

Table 1
Effects of okadaic acid on ATP release evoked by forskolin in the presence and absence of PMA and H-7

Drugs (μM)	*n*	% release of ATP (mean ± S.E.M.)		
		after forskolin		
		5 min	10 min	15 min
Basal	4	79.25 ± 3.07	63.25 ± 5.87	59.75 ± 7.60
Forskolin (1)	7	182.00 ± 11.14	139.00 ± 10.08	112.71 ± 11.18
H-7 (10) + forskolin	4	160.50 ± 13.18	117.50 ± 15.26	102.25 ± 9.26
PMA (0.01) + forskolin	5	148.40 ± 10.48	134.60 ± 22.67	104.60 ± 15.78
Okadaic acid (10) + forskolin	3	98.53 ± 9.72 **	81.30 ± 14.75 *	73.50 ± 12.70
H-7 + okadaic acid + forskolin	5	91.72 ± 6.56 **	82.19 ± 7.71 *	68.81 ± 15.26
PMA + okadaic acid + forskolin	4	174.70 ± 31.00	140.10 ± 6.25	130.53 ± 10.51

Values are expressed as percentage (mean ± S.E.M.) of control (100%) before forskolin. Okadaic acid and other inhibitors (H-7 and PMA) were added to the bath 30 and 35 min, respectively, before forskolin. $^{*}P < 0.05$, $^{**}P < 0.01$ from the forskolin-evoked release.

hibitor, were 35.16 ± 0.72 ($n = 5$) and 39.07 ± 7.76 ($n = 3$) pmol/g wet weight, respectively. Nevertheless, the release of ATP evoked by forskolin was strongly inhibited by the addition of 10 μM okadaic acid. This inhibition by okadaic acid was fully antagonized in the presence of 0.01 μM PMA, an activator of c-kinase, but was unaltered in the presence of H-7, an inhibitor of c-kinase. Both PMA and H-7 per se failed to affect the forskolin-evoked release of ATP.

These results are shown in Table 1.

The contraction enhanced by forskolin was virtually unaltered in the presence of okadaic acid. In addition, PMA and H-7 did not affect the enhanced contraction by forskolin as well (data not shown).

4. Discussion

Previous findings provide evidence that ouabain, a typical cardiotonic, is able to elicit the co-release of ATP and noradrenaline from adrenergic nerves of guinea-pig vas deferens (Katsuragi et al., 1988) and rabbit pulmonary arteries (Katsuragi and Su, 1982). Another type of cardiotonic, forskolin, which increases intracellular cyclic AMP, like isoprenaline, enhances the stimulation-evoked [^{3}H]noradrenaline overflow from sympathetic neurons of the chick (Boehm et al., 1994) and of rat tail artery (Ouedraogo et al., 1994) and guinea-pig urethra (Alberts, 1992). Furthermore, in rat atria under electrical nerve stimulation, forskolin caused an increase in [^{3}H]noradrenaline overflow in the presence of yohimbine (Kazaneitz and Enero, 1992).

In the present study, forskolin and ouabain produced ATP release coupled with an enhanced contraction in guinea-pig atrial segments under electrical stimulation. Both 30 μM W-7, a calmodulin antagonist, and vinblastine, a mitotic inhibitor, strongly reduced the release of ATP evoked by these cardiotonics without affecting the evoked contraction. W-7 hinders [^{3}H]noradrenaline release from digitonin-permeabilized bovine adrenal chromaffin cells (Matsuda et al., 1994). Further, we have found that W-7 as well as trifluoperazine prevent the release of NA evoked by ouabain from guinea-pig vas deferens, probably through an inhibition of Ca^{2+}/calmodulin dependent protein kinase which is involved in exocytosis. Mitotic inhibitors such as vinblastine are well known to inhibit the axoplasmic transport of amine-containing granules in adrenergic nerves (Cheney et al., 1973). Accordingly, the release of ATP evoked by forskolin and ouabain seems to originate from cardiac adrenergic nerves, presumably in an exocytotic manner. *n*-Ethylmaleimide is considered to play a role in blocking the membrane fusion of synaptic vesicles with the presynaptic plasma membrane (Söllner and Rothman, 1994). However, as a SH inhibitor, *n*-ethylmaleimide has a variety of inhibitory effects on cellular functions, e.g., ATP synthesis in mitochondria. As found here, *n*-ethylmaleimide inhibited both ATP release and the contraction induced by forskolin as well as ouabain, reflecting a nonspecific effect via inhibition of SH enzymes. From the findings with *n*-ethylmaleimide, therefore, it seems to be difficult to specify the site of the release of ATP.

It has been hypothesized that the excessive dephosphorylation produced by protein phosphatase inhibitors such as okadaic acid at high concentrations may produce a suppression of neuronal transmission, e.g., via inactivation of presynaptic Ca^{2+} channel proteins (Takuma and Tachibana, 1991; Yanagihara et al., 1991). In a preceding paper (Ogawa et al., 1994), we have presented findings that noradrenaline release evoked by KCl and ouabain from guinea-pig vas deferens is decreased by addition of okadaic acid, and further, the inhibition is overcome by activation of voltage-gated Ca^{2+} channels by Bay K 8644.

In a previous paper (Katsuragi et al., 1993b), forskolin-evoked ATP release was virtually unaffected by prazosin and propranolol, and by guanethidine, at concentrations of 0.3–1.0 μM. Therefore, we guessed that the nucleotide might be released from muscular sites of atrial segments. However, forskolin failed to elicit any ATP release from ventricular segments, which have a much sparser innervation of adrenergic nerves, unlike atrial segments. In addition, from the present evidence obtained by using W-7, vinblastine and okadaic acid, the muscular origin of ATP release considered previously seems to be highly doubtful and, thus, a neuronal origin rather than a non-neuronal origin is more likely, as reported for isoprenaline-evoked ATP release (Tokunaga et al., 1995), although this remains uncertain.

The release of ATP evoked by α, β-methylene ATP from the vas deferens is proposed to be non-neuronal release, because suramin-sensitive release is unaffected by Ca^{2+} removal from the medium (Katsuragi et al., 1991, 1993). Dissimilar to the neuronal release of ATP, the non-neuronal release of ATP evoked by α, β-methylene ATP is not inhibited, but rather shows a tendency to be increased, in the presence of okadaic acid (Ogawa et al., 1994). As shown here, the forskolin-evoked release of ATP was markedly diminished by administration of okadaic acid at a high concentration of 10 μM. The inhibition by the marine toxin of the evoked release of ATP was thoroughly antagonized in the presence of PMA, but not H-7, an activator (Castagna et al., 1982) and inhibitor (Kawamoto and Hidaka, 1984) of protein kinase C, respectively. Thus, the phorbol ester may interfere with the binding of okadaic acid to protein phosphatases by unknown mechanisms. These findings with okadaic acid further strengthen the view that the release of ATP from the

atrial segments evoked by forskolin is of neuronal origin.

In conclusion, since forskolin-evoked ATP release is W-7, vinblastine and okadaic acid-sensitive, forskolin as well as ouabain may preferentially provoke the neuronal release of ATP from cardiac adrenergic nerves. However, the participation of cardiac muscles as the origin of release of ATP could not be definitely excluded because of the broad effects of the test inhibitors used.

Acknowledgements

This work was supported by a Grant-in Aid for Scientific Research (No. 02670102, T.K.) from the Ministry of Education, Sciences and Culture of Japan.

References

Alberts, P. 1992, Subtype classification of the presynaptic α-adrenoceptors which regulate [^{3}H]-noradrenaline secretion in guinea-pig isolated urethra, Br. J. Pharmacol. 105, 142.

Boehm, S., S. Huck, G. Koth, H. Drobny, E. Agneter and E.A. Singer, 1994, α_2-Adrenoceptor-mediated inhibition of electrically evoked [^{3}H]noradrenaline release from chick sympathetic neurons: role of cyclic AMP, J. Neurochem. 63, 146.

Burnstock, G., 1986, Purines as cotransmitters in adrenergic and cholinergic neurones, Prog. Brain Res. 68, 193.

Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa and Y. Nishizuka, 1982, Direct activation of calcium activated, phospholipid-dependent protein kinase C by tumor-promoting phorbol esters, J. Biol. Chem. 257, 7847.

Cheney, D.L., I. Hanin, R. Massarelli, M. Trabucchi and E. Costa, 1973, Vinblastine and vincristine: a study of their action on tissue concentrations of epinephrine, norepinephrine and acetylcholine, Neuropharmacology 12, 233.

Katsuragi, T. and T. Furukawa, 1985, Novel, selective purinoceptor antagonists. Investigation of ATP as a neurotransmitter, Trends Pharmacol. Sci. 6, 337.

Katsuragi, T. and C. Su, 1980, Purine release from vascular adrenergic nerves by high potassium and a calcium ionophore, A-23187, J. Pharmacol. Exp. Ther. 215, 685.

Katsuragi, T. and C. Su, 1982, Release of purines and noradrenaline by ouabain and high KCl from vascular adrenergic nerves, Br. J. Pharmacol. 77, 625.

Katsuragi, T., T. Tokunaga, K. Miyamoto, L. Kuratomi and T. Furukawa, 1988, Norepinephrine and adenosine triphosphate release in different ratio from guinea pig vas deferens by high potassium chloride, ouabain and monensin, J. Pharmacol. Exp. Ther. 247, 302.

Katsuragi, T., T. Tokunaga, S. Ogawa, O. Soejima, C. Sato and T. Furukawa, 1991, Existence of ATP-evoked ATP release system in smooth muscles, J. Pharmacol. Exp. Ther. 259, 513.

Katsuragi, T., K. Shirakabe, O. Soejima, T. Tokunaga, K. Matsuo, C. Sato and T. Furukawa, 1993a, Possible transsynaptic cholinergic neuromodulation by ATP release from ileal longitudinal muscles of guinea pigs, Life Sci. 53, 911.

Katsuragi, T., T. Tokunaga, M. Ohba, C. Sato and T. Furukawa, 1993b, Implication of ATP released from atrial, but not papillary, muscle segments of guinea pig by isoproterenol and forskolin, Life Sci. 53, 961.

Katsuragi, T., S. Ogawa and T. Furukawa, 1994, Contribution of intra and extracellular Ca^{2+} to noradrenaline exocytosis induced by ouabain and monensin from guinea-pig vas deferens, Br. J. Pharmacol. 113, 795.

Kawamoto, S. and H. Hidaka, 1984, 1-(5-isoquinoline sulfonyl)-2-methylpiperazine (H-7) is a selective inhibitor of protein kinase C in rabbit platelets, Biochem. Biophys. Res. Commun. 125, 258.

Kazanietz, M.G. and M.A. Enero, 1992, Role of cyclic AMP in the release of noradrenaline from isolated rat atria. Effect of pretreatment with clenbuterol, Naunyn-Schmied. Arch. Pharmacol. 346, 311.

Kirkpatrik, K. and G. Burnstock, 1987, Sympathetic nerve-mediated release of ATP from the guinea-pig vas deferens is unaffected by reserpine, Eur. J. Pharmacol. 138, 207.

Kranzhöfer, R., M. Haass, T. Kurz, G. Richardt and A. Schömig, 1991, Effect of digitalis glycosides on norepinephrine release in the heart: dual mechanism of action, Circ. Res. 68, 1628.

Matsuda, M., T. Okabe, N. Sugimoto, T. Senda and H. Fujita, 1994, Tetanus toxin and *Clostridium perfringens* enterotoxin as tools for the study of exocytosis, in: Toxins and Exocytosis, Vol. 710, eds. D. Suput and R. Zorec (N.Y. Acad. Sci., New York) p. 94.

Ogawa, S., T. Katsuragi, K. Matsuo and T. Furukawa, 1994, Inhibitory effect of okadaic acid on noradrenaline exocytosis from guinea-pig vas deferens, Neurosci. Lett. 178, 144.

Ouedraogo, S., J.C. Stoclet and B. Bucher, 1994, Effects of cyclic AMP and analogues on neurogenic transmission in the rat tail artery, Br. J. Pharmacol. 111, 625.

Sneddon, P. and G. Burnstock, 1985, ATP as a co-transmitter in rat tail artery, Eur. J. Pharmacol. 106, 149.

Sneddon, P., D.P. Westfall and J.S. Fedan, 1982, Cotransmitters in motor nerves of the guinea-pig vas deferens: electrophysiological evidence, Science 218, 693.

Söllner, T. and J.E. Rothman, 1994, Neurotransmission: harnessing fusion machinery at the synapse, Trends Neurosci. 8, 344.

Su, C., 1983, Purinergic neurotransmission and neuromodulation, Annu. Rev. Pharmacol. Toxicol. 23, 397.

Su, C., G. Burnstock and J.A. Bevan, 1971, [^{3}H]Adenosine triphosphate: release during stimulation of enteric nerves, Science 173, 337.

Takuma, T. and T. Ichida, 1991, Okadaic acid inhibits amylase exocytosis from protid acini stimulated by cyclic AMP, FEBS Lett. 285, 124.

Tokunaga, T., T. Katsuragi and T. Furukawa, 1995, ATP release evoked by isoprenaline from adrenergic nerves of guinea pig atrium, Neurosci. Lett. 186, 95.

Von Kügelgen, I. and K. Starke, 1985, Noradrenaline and adenosine triphosphate as co-transmitters of neurogenic vasoconstriction in rabbit mesenteric artery, J. Physiol. (London) 367, 435.

Von Kügelgen, I. and K. Starke, 1991, Noradrenaline-ATP co-transmission in the sympathetic nervous system. Trends Pharmacol. Sci. 12, 319.

Yanagihara, N., Y. Toyohira, Y. Koda, A. Wada and F. Izumi, 1991, Inhibitory effect of okadaic acid on carbachol-evoked secretion of catecholamines in cultured bovine adrenal medullary cells, Biochem. Biophys. Res. Commun. 174, 77.