ATP modulates the efferent function of capsaicin-sensitive neurones in guinea-pig isolated atria

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1 The effect of adenosine triphosphate (ATP) and its stable analogues, α,β-methylene-ATP and β,γ-methylene-ATP, on the efferent function of capsaicin-sensitive non-adrenergic, non-cholinergic (NANC) nerves was tested in guinea-pig isolated atria.

2 Transmural nerve stimulation of atria isolated from reserpine-pretreated guinea-pigs, in the presence of 1 μM atropine and 0.3 μM CGP 20712A, induced a transient positive inotropic effect attributable to calcitonin gene-related peptide (CGRP) release from NANC nerve endings.

3 ATP (1–30 μM) concentration-dependently reduced the cardiac response to transmural nerve stimulation, without affecting the inotropic response to 10 nM exogenous CGRP. The inhibitory effect of ATP was competitively antagonized by the P_1-purinoceptor antagonist, 8-phenyltheophylline (8-PT, 1 μM), but was unaffected by the P_2-purinoceptor antagonist, suramin (100 μM).

4 β,γ-Methylene-ATP in the same concentration range as ATP, inhibited the cardiac response to transmural nerve stimulation. The inhibitory effect of β,γ-methylene ATP was antagonized by 1 μM 8-PT. The desensitizing agonist for P_2-purinoceptors, α,β-methylene ATP did not induce any inhibitory effect either on the cardiac response to transmural nerve stimulation or on the inhibitory effect curve for ATP.

5 The inhibitory effect of ATP on the NANC neurotransmission was inconsistently modified in the presence of 10 μM α,β-methylene-adenosine diphosphate, an inhibitor of the 5'-ectonucleotidases.

6 These results demonstrate that ATP modulates the efferent function of cardiac NANC nerve endings through prejunctional inhibitory receptors belonging to the P_1 type. The metabolic conversion of ATP to adenosine does not seem to be a pre-requisite for the ATP agonist activity.

Keywords: ATP; purinoceptors; NANC neurotransmission; capsaicin-sensitive neurones; guinea-pig atria

Introduction

Primary sensory neurones can have both an afferent and an efferent function due to the release of their transmitter content at the peripheral level (Maggi & Meli, 1988; Burnstock, 1990). In the cardiac tissue the presence of capsaicin-sensitive non-adrenergic, non-cholinergic (NANC) nerves has been demonstrated by immunohistochemical (Hougland et al., 1986; Wharton et al., 1986) and pharmacological studies (Saito et al., 1986). The efferent function of the capsaicin-sensitive primary sensory nerves can be activated by different stimuli, including electrical field stimulation (Maggi & Meli, 1988). The antidromic invasion of capsaicin-sensitive neurones in cardiac tissue induces a positive inotropic effect, mainly due to calcitonin gene-related peptide (CGRP) release (Miyauchi et al., 1987; Saito et al., 1987; Maggi et al., 1991). It has been reported that several substances, including opioid peptides (Mantelli et al., 1989; 1990a; Giuliani et al., 1990), α-adrenoceptor agonists (Mantelli et al., 1990b), neuropeptide Y (Giuliani et al., 1989a) and the peptide galanin (Giuliani et al., 1989b) modulate the efferent function of capsaicin-sensitive neurones in cardiac tissue. Moreover it has been recently demonstrated that endogenous adenosine, released during the transmural nerve stimulation of guinea-pig isolated atria, exerts an inhibitory control of NANC neurotransmission through prejunctional A_1 receptors (Rubino et al., 1990; 1991).

Adenosine shares with adenine nucleotides, including adenosine 5'-triphosphate (ATP), its inhibitory control of the neurotransmission process both in the central nervous system and in peripheral organs by reducing the transmitter release and altering the responses of the target organ to the transmitter released (Fredholm & Hedqvist, 1980; Williams, 1987). In the heart, adenosine and ATP, released during nerve activity and during the contractile response of the muscle cells to nerve stimulation or other stimuli, play a crucial role in the

regulation of sympathetic neurotransmission by acting on specific cell membrane receptors (Hedqvist & Fredholm, 1979; Fredholm & Hedqvist, 1980).

A regulatory role for adenosine has been demonstrated on the cardiac NANC neurotransmission (Rubino et al., 1990); however no information is available on the role of ATP in the modulation of NANC neurotransmission in the heart. Therefore in the present study we have investigated the effect of ATP on the efferent function of primary sensory neurones in guinea-pig isolated atria and have tested whether ATP was active per se or by its breakdown to adenosine. Moreover, since the purinoceptors have been classified into the P_1 and P_2 subtypes (Burnstock, 1978), we have also characterized the kind of purinoceptors involved in the inhibitory action of ATP.

Methods

Experimental procedure

The experimental model used for this study has been previously described in more detail (Mantelli et al., 1989). Atrial preparations were isolated from male guinea-pigs (200–300 g) pretreated with reserpine (5 mg kg^{-1}, i.p.) 24 h before the experiment. Preparations were mounted vertically in an organ bath containing Tyrode solution of the following composition (mM): NaCl 115, KCl 4.7, CaCl$_2$ 1.8, MgSO$_4$ 1.2, KH$_2$PO$_4$ 1.2, NaHCO$_3$ 25 and glucose 10, oxygenated with 95% O$_2$ and 5% CO$_2$ and kept at the constant temperature of 30°C. The atria were stretched until the maximum force of contraction was reached and then maintained at this length throughout the experiment. The isometric contraction was recorded by an isometric transducer and a d.c. preamplifer on a pen recorder (Battaglia Rangoni KV 135) and on a dual beam oscilloscope (Tektronix D 13).

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Preparations were driven electrically at a constant rate (4 Hz) by punctate electrodes connected with a pulse generator (Tektronix type 161 pulse generator). Transmural nerve stimulation was performed by applying trains of field pulses (50–100 mA intensity; 1 ms duration) through two platinum plates, parallel to the preparations, connected with a second pulse generator (MARB 82/2/200). Trains of field pulses, consisting of two pulses for each of 40 consecutive contractions, were applied during the absolute refractory period in order to avoid interference with the normal rhythm of contractility. The parasympathetic component of the response to field stimulation was eliminated by the addition of 1 μM atropine to the Tyrode solution. Moreover 0.3 μM CGP 20712A (a β_1-adrenoceptor selective antagonist) was added to the solution in order to eliminate the residual adrenergic component of the response. As already described, in these experimental conditions the cardiac response to transmural nerve stimulation was abolished by the treatment with the peptidergic toxin capsaicin (Mantelli et al., 1989) and was concentration-dependently reduced by the CGRP receptor antagonist, hCGRP (8–37), used at micromolar concentrations (Rubino et al., 1991), thus confirming the hypothesis that NANC neurotransmission was being evaluated. After a period of equilibration of at least 60 min, trains of field pulses were applied at 15 min intervals. The response to transmural nerve stimulation was evaluated as the difference between the maximum force of contraction developed after transmural nerve stimulation and the basal contractility (ΔFc). Since the response to each train remained reproducible for many consecutive tests, it was possible to obtain cumulative concentration-effect curves for the agonists. Increasing concentrations of the agonists were added to the bathing solution after a control train; transmural nerve stimulation was then applied again after 5 min contact with all of the agonists tested. Only one concentration-effect curve was usually obtained in each preparation. The control response to field stimulation was taken as 100% and the effect of the agonist tested, alone and in the presence of antagonist, was evaluated as percentage inhibition of the control response. The incubation periods for suramin and 8-phenyltheophylline (8-PT) were 10 and 20 min of contact, respectively, before treating the atrial preparation with the agonist.

Drugs

Adenosine triphosphate (ATP), α,β-methylene-ATP, β,γ-methylene-ATP, α,β-methylene-ADP, suramin, rat calcitonin gene-related peptide (CGRP) and reserpine were purchased from Sigma Chemical Co., St. Louis, U.S.A. Atropine sulphate was obtained from BDH, Poole, England. 8-Phenyltheophylline (8-PT) was furnished by Calbiochem, San Diego, California, U.S.A.; 2-hydroxy-5-{2-[hydroxy-3-(4-[(1-methyl-4-trifluoromethyl)1H-imidazol-2-yl-]-phenoxy)-propyl]aminoethoxy}-benzamide (CGP 20712A) was kindly supplied by Ciba Geigy, Basel, Switzerland.

A stock solution of 10 mM 8-PT was made up in 80% ethanol containing 0.2 M NaOH; further dilutions were made in distilled water.

Data evaluation

The data shown in the test and in the figures are reported as mean values ± s.e.mean. Statistical analysis was performed by means of Student's t test for unpaired data.

Results

Effect of ATP alone and in the presence of 8-phenyltheophylline (8-PT) or suramin

Transmural nerve stimulation of guinea-pig atria, isolated from reserpine-pretreated animals, in the presence of 1 μM

atropine and 0.3 μM CGP 20712A, increased cardiac contractility from 341.8 ± 18.6 to 520.9 ± 26.0 mg (n = 36). The maximum inotropic response to transmural nerve stimulation was reached within about 40 s and cardiac contractility declined to basal values in about 5 min. A recording taken from a typical experiment showing the cardiac response to transmural nerve stimulation in control conditions and in the presence of 3 and 30 μM ATP is shown in Figure 1. It is evident that the addition of 1 μM 8-PT to the bathing solution reversed the inhibitory effect of 30 μM ATP. ATP (1–30 μM) reduced the cardiac response to transmural nerve stimulation in a concentration-dependent manner (Figure 2). The maximum effect consisted of 52.7 ± 3.4% inhibition of control response to transmural nerve stimulation and was almost
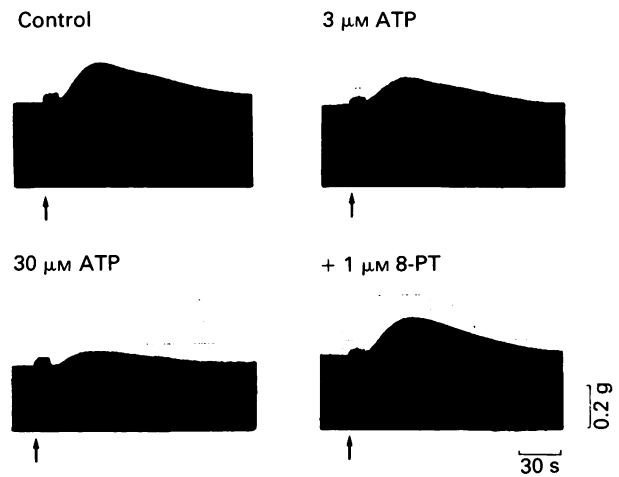
Figure 1 Records taken from a typical experiment showing the response to transmural nerve stimulation on atria obtained from reserpine-pretreated guinea-pig, in the presence of 1 μM atropine and 0.3 μM CGP 20712A. The cardiac response to transmural nerve stimulation before and after treatment with increasing concentrations (3 and 30 μM) of ATP is shown. The addition to the bathing solution of 1 μM 8-phenyltheophylline (8-PT) reversed the inhibitory effect of 30 μM ATP. The arrows indicate the beginning of transmural nerve stimulation.
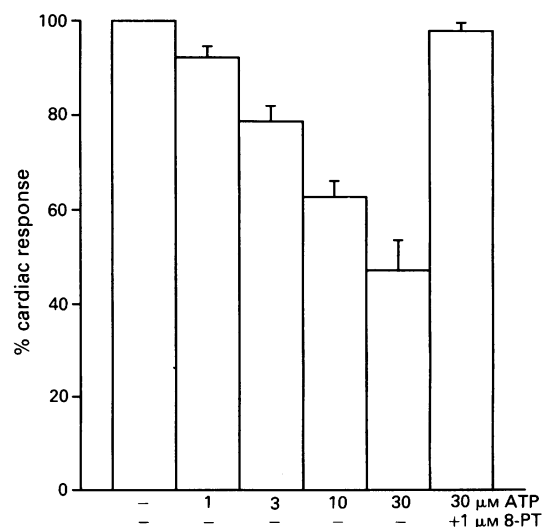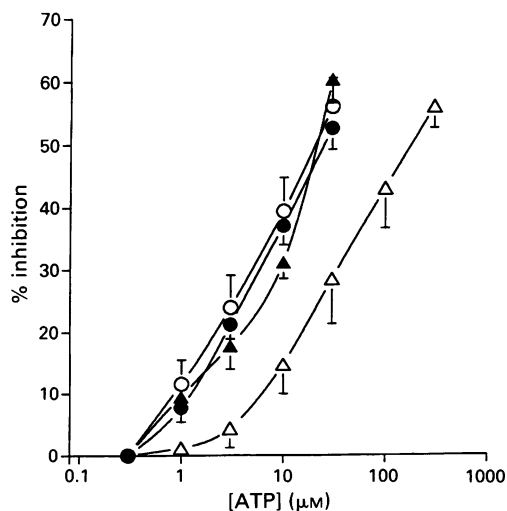

Figure 2 Concentration-dependent inhibitory effect of ATP on the cardiac response to transmural nerve stimulation. The increase in cardiac contractility induced by transmural nerve stimulation was taken as 100%. 8-Phenyltheophylline (8-PT) was added to the bathing solution in the presence of the highest ATP concentration. Means of 6 experiments with s.e.mean shown by vertical bars.

completely abolished by 1 μM 8-PT (Figure 2). At concentrations of 10 and 30 μM, ATP had a slight direct negative inotropic effect; the basal contractility was reduced from 341.6 ± 56.9 to 309.6 ± 54.4 mg in the presence of 10 μM ATP (n = 6) and to 266.6 ± 50.7 mg, in the presence of 30 μM ATP (n = 6). Although a 100% inhibition of the cardiac response to transmural nerve stimulation was never reached, higher concentrations of ATP were not tested to prevent the direct negative inotropic effect from interfering with the evaluation of the ATP reduction in cardiac response to electrical stimulation.

The concentration-inhibitory effect curve for ATP was not affected by a concentration of suramin (100 μM) (Figure 3) that fully antagonizes P₂-purinoceptor activation. On the other hand, the control curve for ATP was shifted to the right in a parallel fashion by 1 μM 8-PT (Figure 3). The basal cardiac contractility was affected neither by suramin not by 8-PT. Whereas 1 μM 8-PT increased the control response to transmural nerve stimulation by about 30% (ΔFc was 193 ± 16 and 243 ± 22.3 mg in the absence and presence of 8-PT, respectively) as already reported (Rubino et al., 1990), suramin did not affect the cardiac response to transmural nerve stimulation.

Effect of ATP on the cardiac response to exogenous calcitonin gene-related peptide

At the highest concentration tested, ATP did not modify the positive inotropic effect of exogenous CGRP, thus suggesting a prejunctional inhibitory effect of ATP. A concentration of CGRP (10 μM) was selected that produced a positive inotropic response superimposable on that obtained with transmural nerve stimulation of atrial preparations. When 10 nM CGRP was added to the bathing solution in the absence and in the presence of 30 μM ATP the cardiac contractility increased from 329.2 ± 20.4 to 516.0 ± 31.6 mg (n = 10) and from 263.5 ± 34.3 to 441.7 ± 36.7 mg (n = 8), respectively. As reported above, 30 μM ATP reduced the basal contractility by about 20%, but the preparations were still able to respond to the exogenously applied CGRP, the ΔFc being 187.4 ± 19.7 and 178.3 ± 27.1 mg in the presence and absence of ATP, respectively.

Effects of stable analogues of ATP, α,β-methylene-ATP and β,γ-methylene-ATP

The effect of slowly degradable ATP analogues on the cardiac response to transmural nerve stimulation was tested. α,β-Methylene-ATP, which first activates and then desensitizes selectively the P₂-purinoceptor at micromolar concentrations did not show any effect either on the cardiac response to transmural nerve stimulation or on the basal contractility (data not shown). Furthermore, in preparations exposed to a desensitizing concentration of α,β-methylene-ATP (50 μM) for at least 20 min the concentration-inhibitory effect curve for ATP was not statistically different from that for ATP alone (Figure 3). These observations argue against the involvement of P₂-purinoceptors in mediating the effect of ATP.

β, γ-Methylene-ATP, in the same concentration range as ATP (1–30 μM), concentration-dependently reduced the cardiac response to transmural nerve stimulation (Figure 4), without affecting basal cardiac contractility in a significant way. The maximum inhibitory effect reached with 30 μM β,γ-methylene-ATP consisted of 58.8 ± 4.5% inhibition of the control response to transmural nerve stimulation, and was not statistically different from the maximum inhibitory effect of ATP. The inhibitory effect of β,γ-methylene-ATP at the highest concentration tested (30 μM) was completely antagonized by 1 μM 8-PT (Figure 4).

Effect of ATP in the presence of α,β-methylene-ADP

The effect of ATP in the presence of the inhibitor of 5'-ectonucleotidases, α,β-methylene-ADP, used at a concentration fully active in inhibiting the enzyme in the cardiac tissue was evaluated. The inhibitory effect of increasing concentrations of ATP in the presence of 10 μM α,β-methylene-ADP, compared to the effect of ATP in control conditions, is shown in Figure 5. ATP partially lost its inhibitory effect on the cardiac response to transmural nerve stimulation only at the lower concentrations used (1 and 3 μM). However it was still able to reduce the response to transmural nerve stimulation by 34.9 ± 7.2 and 56.0 ± 9.1% at 10 and 30 μM, respectively. The effect of 30 μM ATP alone and in the presence of α,β-methylene-ADP was reversed by 1 μM 8-PT (not shown). α,β-Methylene-ADP did not have any effect either on basal con-

Figure 3 Concentration-inhibitory effect curves for ATP alone (●; n = 6) and in the presence of 100 μM suramin (○; n = 10), 1 μM 8-phenyltheophylline (8-PT; △; n = 6) and 50 μM α,β-methylene-ATP (▲; n = 6) on the cardiac response to transmural nerve stimulation. The response to transmural nerve stimulation obtained before drug addition was considered as 100% and the effect of ATP, alone and in the presence of suramin, 8-PT or α,β-methylene ATP, was evaluated as percentage inhibition of the control response.

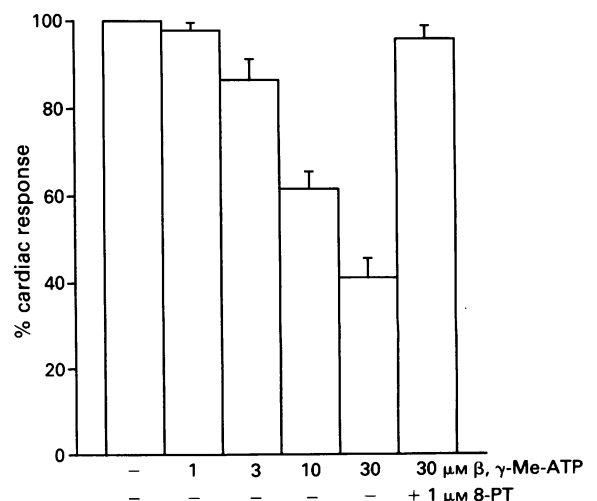**Figure 4** Concentration-dependent inhibitory effect of β,γ-methylene-ATP (β,γ-Me-ATP) on the cardiac response to transmural nerve stimulation. The increase in cardiac contractility induced by transmural nerve stimulation was taken as 100%. 8-Phenyltheophylline (8-PT) was added to the bathing solution in the presence of the highest β,γ-methylene-ATP concentration. Means of 4 experiments with s.e.mean shown by vertical bars.
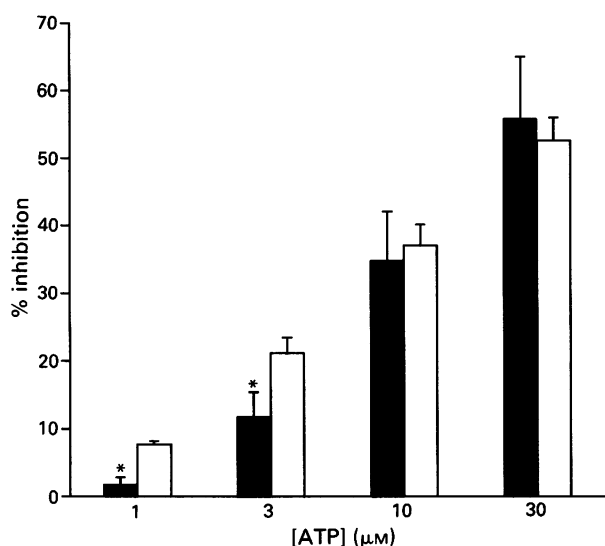
Figure 5 Inhibitory effect of increasing concentrations of ATP alone (n = 6, open columns) and in the presence of 10 μM α,β-methylene-ADP (n = 6, solid columns). The response to transmural nerve stimulation obtained before drug addition was taken as 100% and the effect of ATP alone and in the presence of α,β-methylene-ADP was evaluated as percentage inhibition of the control response. * for at least P < 0.05.

tractility or on the control response to transmural nerve stimulation.

Discussion

Evidence has been produced indicating that purines are released as cotransmitters or neuromodulators following nerve stimulation in various peripheral organs, including the heart (Fredholm & Hedqvist, 1980). Besides the neuronal origin for adenosine and ATP, purine release has been demonstrated to occur in cardiac muscle cells, in response to nerve stimulation or other noxious stimuli, such as hypoxia or ischaemia (Forrester & Williams, 1977; Rubio & Berne, 1985). This last condition has been reported to activate the efferent function of capsaicin-sensitive NANC cardiac neurones (Franco-Cereceda et al., 1989). In addition, the presence of quinacrine-positive fibres containing ATP has been demonstrated in guinea-pig atria (Crowe & Burnstock, 1982). Recent evidence indicates the presence of receptors for ATP on intracardiac neurones in culture, supporting the hypothesis that both adenosine and adenine nucleotides play a role in the regulation of cardiac function (Allen & Burnstock, 1990). Actually adenosine and ATP regulate adrenergic neurotransmission in the heart through pre- and postsynaptic effects mediated by specific receptors (Hedqvist & Fredholm, 1979; Fredholm & Hedqvist, 1980; Burnstock, 1980). Adenyl compounds may also have a physiological modulatory action on acetylcholine release from cholinergic nerve terminals in the heart (Burnstock, 1980).

In addition to being controlled by sympathetic adrenergic and parasympathetic cholinergic nerves, cardiac functions may be controlled by NANC nerves, present in the heart of several species including man (Franco-Cereceda, 1988). We have recently demonstrated that endogenous adenosine regulates cardiac NANC neurotransmission through prejunctional inhibitory receptors belonging to the A_1 subtype (Rubino et al., 1990; 1991). In the present study we demonstrate that the adenosine nucleotide ATP exerts an inhibitory control of cardiac NANC neurotransmission in guinea-pig isolated atria, which seems to be mediated by prejunctional receptors. ATP, at the highest concentrations tested, induced a slight negative inotropic effect, as previously observed by other authors

(Burnstock & Meghji, 1981). However, the maximum inhibitory effect of cardiac response to transmural nerve stimulation induced by 30 μM ATP was more than two times greater than the direct negative inotropic effect induced by the same concentration of ATP. Moreover ATP, at the highest concentration tested, did not modify the inotropic effect of exogenous CGRP, thus indicating a prejunctional activity. The inhibitory effect of ATP on cardiac NANC neurotransmission appeared to be mediated by receptors sensitive to methylxanthines, since 8-PT, a potent P_1-purinoceptor antagonist (Griffith et al., 1981), competitively antagonized the inhibitory effect of ATP. On the other hand, suramin, a reversible antagonist at the P_2-purinoceptor (Dunn & Blakeley, 1988; Hoyle et al., 1990), did not affect ATP inhibitory activity in our experimental model. Thus our results indicate that the inhibitory effect of ATP on cardiac NANC neurotransmission is mediated by prejunctional P_1-purinoceptors. This hypothesis is further supported by results obtained with the selective P_2-purinoceptor agonist, α,β-methylene-ATP, which was used as a tool for revealing the presence of P_2 purinoceptors, because of its ability to activate and then desensitize this kind of receptor (Kasakov & Burnstock, 1983). If P_2-purinoceptors were involved in ATP activity it would not be possible to observe the inhibitory effect of ATP, after challenging the atrial preparations with the desensitizing agonist, α,β-methylene-ATP. However in our experimental model α,β-methylene ATP did not show either any direct effect or reduction of ATP inhibitory activity, thus excluding the involvement of a P_2-purinoceptor in the ATP effects.

In the heart, ATP is rapidly hydrolyzed to form 5'-AMP and adenosine (Hopkins, 1973), the conversion of 5'-AMP by 5'-ectonucleotidases being the final step in the hydrolysis of ATP to adenosine (Pearson et al., 1980). This enzyme is inhibited by α,β-methylene-ADP (Bruns, 1980). It could be suggested that ATP must be converted to adenosine in order to cause its presynaptic action on the cardiac NANC neurotransmission. However, our observation that in the presence of the enzyme inhibitor, α,β-methylene-ADP, only the lowest concentrations of ATP tested partly lost their inhibitory effect, indicates that conversion to adenosine is not a prerequisite for the ATP agonist activity at the P_1-purinoceptor. These findings suggest that ATP can be active by itself at this receptor, as already shown in the guinea-pig atria (Burnstock & Meghji, 1981; Collis & Pettinger, 1982). Further evidence that the breakdown of ATP is not necessary for activation of the P_1-purinoceptor is provided by the observation that the hydrolysis resistant β,γ-methylene isostere of ATP showed potent P_1-purinoceptor agonist activity. Actually, β,γ-methylene-ATP was active at the same concentrations as ATP in inhibiting NANC neurotransmission and its inhibitory activity was reversed by 8-PT. Moreover, at the concentrations tested, β,γ-methylene-ATP was less active than ATP in exerting the direct negative inotropic effect, as previously reported by Burnstock & Meghji (1981). The agonist activity of ATP analogues, including β,γ-methylene-ATP, at P_1-purinoceptors, antagonized by xanthines has already been demonstrated by other authors (Burnstock & Meghji, 1981; Wiklund & Gustaffson, 1986).

In conclusion, our findings provide the first evidence that ATP inhibits the efferent function of capsaicin-sensitive NANC neurones in cardiac tissue. Although it was not possible to support this evidence with measurements of CGRP release, the findings clearly point out a prejunctional site of action, in which P_1 purinoceptors are involved.

Similar results have been obtained in another peripheral preparation, the guinea-pig bronchi by Kamikawa & Shimo (1989). In this preparation, electrical field stimulation induced a noncholinergic functional response due to substance P release, which was inhibited by both ATP and adenosine through prejunctional P_1-purinoceptors.

Furthermore, we have also demonstrated that the degradation of ATP to adenosine by ectoenzymes does not seem to be a prerequisite for activation of the P_1-purinoceptors, although

a part of the ATP effect might be attributable to its conversion to adenosine. Thus endogenous adenosine, as already reported (Rubino *et al.*, 1990), and ATP, as demonstrated in the present study, regulate the peripheral function of capsaicin-sensitive cardiac neurones. The potential interplay between the endogenous purine nucleosides and nucleotides seems to represent a complex and ductile mechanism by which the efferent function of NANC neurotransmission can be regulated.

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