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2′,3′-O-(2,4,6- trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP) – a nanomolar affinity antagonist at rat mesenteric artery P2X receptor ion channels

[1]C.J. Lewis, [2]A. Surprenant & [1,3]R.J. Evans

[1]Department of Cell Physiology & Pharmacology, Medical Sciences Building, University of Leicester, University Road, Leicester LE1 9HN, U.K. and [2]Geneva Biomedical Research Institute, 14 chemin des Aulx, Plan Les Ouates, Geneva, Switzerland

1 P2X receptor activation by α,β-meATP evoked inward currents in acutely dissociated rat mesenteric artery smooth muscle cells and contractions of whole artery rings.

2 The selective $P2X_1$ and $P2X_3$ receptor antagonist TNP-ATP inhibited P2X receptor mediated inward currents in response to 3 μM α,β-meATP (an $\sim EC_{90}$ concentration) with an IC_{50} of $\sim$2 nM. This provides further evidence that the P2X receptor underlying membrane depolarisation associated with P2X receptor activation can be accounted for by the expression of $P2X_1$ receptors.

3 TNP-ATP inhibited α,β-meATP induced contractions with an IC_{50} of $\sim$30 μM and had non-specific effects on smooth muscle contraction.

4 The reduced potency of TNP-ATP in whole tissue experiments probably reflects the breakdown of TNP-ATP by nucleotidases. Thus, TNP-ATP is of limited use in whole tissue experiments as a P2X receptor antagonist.

Keywords: P2X receptors; ATP; artery; contractions; electrophysiology; antagonist

Introduction

P2X receptor mediated constrictions in response to sympathetic nerve stimulation, and the exogenous applications of purinergic agonists have been reported for a variety of arteries e.g. (Kennedy *et al*., 1986; Evans & Surprenant, 1992). Seven members of the P2X ligand–gated ion channel family ($P2X_{1-7}$) have been identified at the molecular level and characterised (Collo *et al*., 1996; Surprenant *et al*., 1996). The properties of vascular smooth muscle P2X receptors can be accounted for by the expression of $P2X_1$ receptors (Evans & Surprenant, 1996). However the lack of subtype selective P2X receptor antagonists has frustrated the study and direct classification of native P2X receptors. Recent studies have shown that 2′,3′-O-(2,4,6- trinitrophenyl) ATP (TNP-ATP), in the nanomolar range, can act as a selective antagonist at recombinant $P2X_1$ and $P2X_3$ homomeric and $P2X_{2/3}$ heteromeric receptors (Virginio *et al*., 1998). $P2X_3$ receptors are thought to be expressed only by sensory neurons (Evans & Surprenant, 1996) and thus TNP-ATP may be useful as a selective antagonist to determine the role of $P2X_1$ receptors in the control of blood flow. In this study we have tested whether TNP-ATP is an antagonist at rat mesenteric artery P2X receptors.

Methods

Male wistar rats (250–300 g) were killed by cervical dislocation and carotid exsanguination. The mesentery was removed and second and third order mesenteric arteries dissected. For contraction experiments mesenteric artery rings were mounted in a Mulvany myograph using standard procedures (vessel diameter 244 ± 10 μm, $n = 42$) (Lagaud *et al*., 1996). Artery rings were superfused at 2 ml.min^{-1} with a physiological solution of the following composition (mM); NaCl 150, KCl, 5, HEPES, 10, $CaCl_2$ 2.5, $MgCl_2$ 1, pH was adjusted to 7.3 with NaOH. Drugs were added to the superfusate to give the required final concentration. α,β-meATP evoked transient contractions of the mesenteric artery that faded and returned to baseline values during the continued presence of agonist (Figure 1a). When applied at 30 min intervals α,β-meATP evoked reproducible responses. Antagonists were pre-superfused for 15–30 min and then added concomitantly with agonist.

Acutely dissociated smooth muscle cells were prepared using an overnight papain digestion (Evans & Kennedy, 1994). Cells were plated onto glass coverslips and superfused at 2 ml.min^{-1} with physiological solution and drugs were applied rapidly using a U-tube perfusion system (Evans & Kennedy, 1994). Whole cell or amphotericin permeabilised patch recordings were made with an Axopatch 200A amplifier and data collected using pClamp 6 software (Axon Instruments, U.S.A.). Holding potential was -60 mV. Electrodes were filled with an internal solution of the following composition (mM); potassium gluconate 140, NaCl 5, HEPES 10, EGTA 9, pH was adjusted to 7.3 with KOH. Reproducible responses to α,β-meATP (3 μM $\sim EC_{90}$, 100–500 ms pulse duration) could be recorded in about 70% of cells when a 5 min interval was given between agonist applications (the remainder showed a significant run-down of responses and were not used in this study). Antagonists were added to the superfusate for 5 min before the co-application of antagonist plus α,β-meATP.

Data are reported throughout as mean $\pm$ s.e.m., n = number of observations. Concentration response data for agonists were fitted by the least squares method with the equation; response $= \alpha[A]^H/([A]^H + [A_{50}]^H)$ where α and H are the asymptote and Hill coefficient, [A] is the agonist concentration, A_{50} is the agonist concentration producing 50% of the

[3] Author for correspondence.

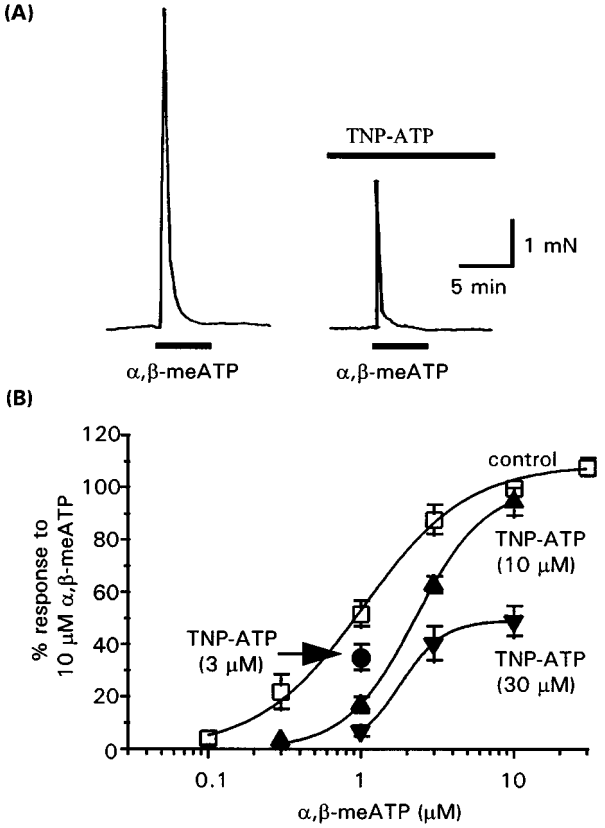

Figure 1 Effects of TNP-ATP on P2X receptor mediated contractions of rat mesenteric artery. (A) constrictions evoked by 10 μM α,β-meATP and inhibition by TNP-ATP (30 μM). Drugs were applied for period shown by bars. TNP-ATP was pre-superfused for 20 min before concomitant application of α,β-meATP. (B) Concentration-response relationship for α,β-meATP (□) and inhibition by TNP-ATP (3 μM ●, 10 μM ▲, 30 μM ▼). ($n=3-6$ for each point).

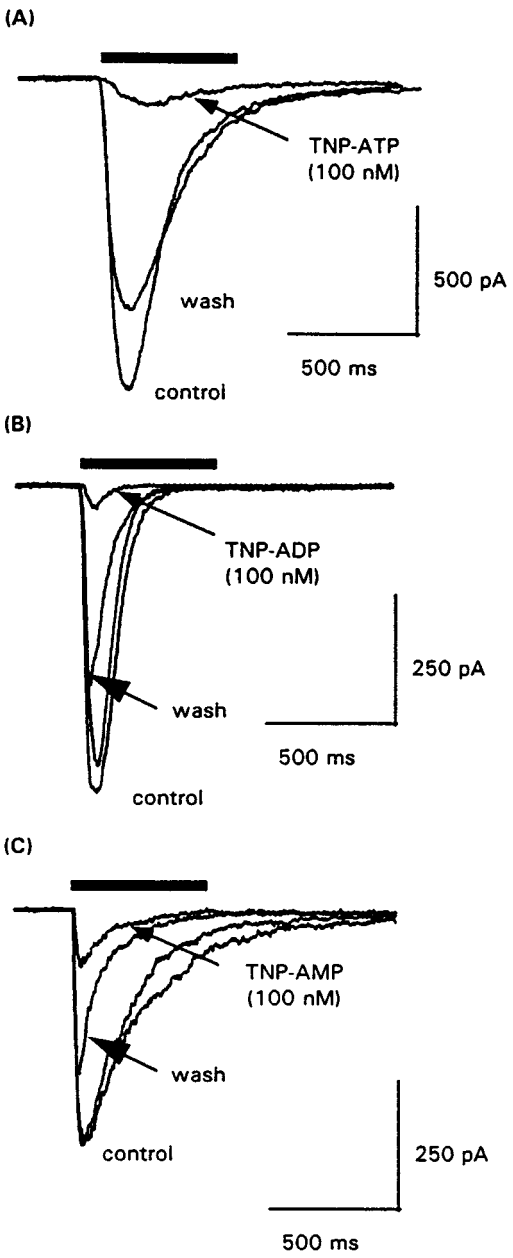

Figure 2 Antagonism of P2X receptor mediated inward currents by TNP analogues. Inhibition of inward currents evoked by 3 μM α,β-meATP (500 ms, indicated by bar) by (A) TNP-ATP (100 nM), (B) TNP-ADP (100 nM) and (C) TNP-AMP (100 nM). Inhibition by TNP analogues was reversed on washout. In B and C two control responses are shown evoked prior to antagonist application.

maximum response (the EC_{50} value). Inhibition of responses to α,β-meATP by TNP-ATP were fit with the same equation where A_{50} corresponds to the concentration of TNP-ATP required to inhibit the response by 50% (the IC_{50} value).

Drugs α,β-methylene ATP (Sigma), 2′3′-O-(2,4,6- trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP), 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-diphosphate (TNP-ADP) and 2′,3′O-(2,4,6- trinitrophenyl) adenosine 5′-monophosphate (TNP-AMP) (Molecular probes).

Results

The metabolically stable ATP analogue α,β-meATP evoked concentration dependent contractions of rat mesenteric artery rings (EC_{50} ~1 μM). The $P2X_1$ receptor antagonist TNP-ATP (3–30 μM) inhibited α,β-meATP evoked responses (Figure 1A,B). 10 μM TNP-ATP gave an essentially parallel shift to the right in the concentration response relationship to α,β-meATP. At 30 μM, TNP-ATP antagonism was non-competitive with a clear depression of the maximal response to α,β-meATP. The inhibition of α,β-meATP responses was reversed following washout. TNP-ADP and TNP-AMP (both 30 μM) had no effect on responses to 10 μM α,β-meATP ($106\pm10.9\%$, $n=6$ and $102.5\pm11\%$, $n=4$ of control responses respectively).

Noradrenaline (10 μM) evoked contractions of equivalent magnitude to 10 μM α,β-meATP. TNP-ATP (30 μM) inhibited responses to 10 μM noradrenaline by $40\pm5.5\%$ ($n=3$). These results indicate that this concentration of TNP-ATP has non-selective actions to block arterial contraction.

The results showing the lack of effect of TNP-ATP in whole tissue studies taken on their own would imply that $P2X_1$-like receptors do not mediate the α,β-meATP mediated responses in the rat mesenteric artery. However, the pharmacological characterisation of P2X receptors in whole tissues has been complicated by the fact that a number of purines are metabolically unstable and thus their true potencies can be underestimated. For example the EC_{50} value for ATP in evoking responses is reduced by up to two orders of magnitude when ectonucleotidase activity is inhibited (Trezise *et al.*, 1994; Crack *et al.*, 1995). These problems of metabolic breakdown can be overcome in patch clamp studies on dissociated smooth

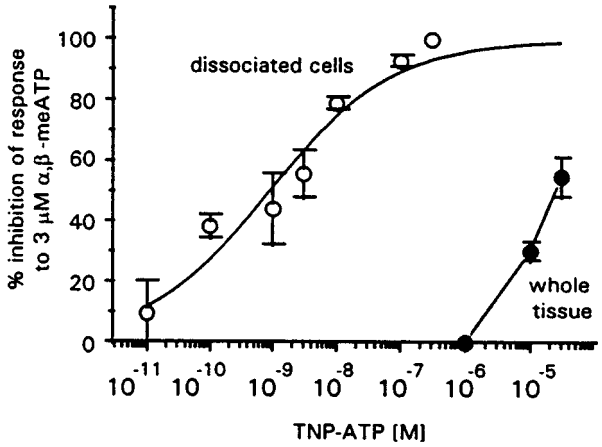

Figure 3 Comparison of antagonist potency of TNP-ATP at P2X receptors measured in whole artery and dissociated smooth muscle cells. Plots show inhibition of responses to 3 μM α,β-meATP recorded from acutely dissociated smooth muscle cells and in contraction studies ($n=3-6$).

muscle cells where drugs can be applied rapidly under concentration clamp conditions (Evans & Kennedy, 1994). Therefore, to determine whether metabolism of TNP-ATP and its derivatives in whole tissue studies could account for their apparent low potency we tested the effects of TNP-ATP on α,β-meATP evoked P2X receptor mediated currents in acutely dissociated rat mesenteric artery smooth muscle cells. α,β-meATP evoked rapidly inactivating P2X receptor mediated inward currents (EC_{50} ~ 1 μM, unpublished observations). In contrast to contraction studies TNP-ATP, TNP-ADP and TNP-AMP (all 100 nM) inhibited inward currents in response to 3 μM α,β-meATP ($\sim EC_{90}$ concentration) by, $93\pm2\%$, 90.5 ± 2.7 and 86.7 ± 4 respectively ($n=3-4$) (Figure 2). These effects were reversed on washout. Thus when TNP-ATP was applied under concentration clamp conditions responses to α,β-meATP (3 μM) were inhibited in a concentration dependent manner with an IC_{50} of ~ 2 nM (Figure 3). In comparison, in whole tissue studies where applied drugs are subject to the actions of nucleotidases, the IC_{50} for TNP-ATP as an antagonist of P2X receptor mediated contractile responses was ~ 30 μM (Figure 3).

Nucleotidase activity is inhibited by reductions in magnesium or pH (Trezise *et al*., 1994; Todorov *et al*., 1997). Therefore we undertook a series of experiments to determine if a reduction in magnesium concentration or pH could be used to reduce nucleotidase activity in rat mesenteric artery rings. An increase in the potency of ATP would indicate an inhibition of nucleotidase activity. Under normal conditions the EC_{50} for ATP was ~ 300 μM ($n=6$), the threshold for evoking contraction or the EC_{50} for ATP were essentially unchanged when experiments were repeated in nominally magnesium free solution. Reducing the pH from 7.3 to 5.4 reduced the magnitude of contraction to ATP and α,β-meATP by 80–95% (a similar pH dependent reduction in the amplitude of $P2X_1$ receptor mediated responses has been reported and is associated with a change in the apparent potency of ATP for the receptor, Stoop *et al*., 1997). These results suggest that low pH or magnesium solutions cannot be used to reduce nucleotidase activity in this preparation and therefore could not be used to test whether TNP-ATP is metabolised by nucleotidases in whole tissue studies.

Discussion

This study shows that TNP-ATP is a highly potent antagonist of P2X receptor mediated inward currents in acutely dissociated rat mesenteric artery smooth muscle cells. P2X receptor mediated ionic currents underlie the contractions evoked by α, β-meATP in rat mesenteric arteries. Calcium may enter the cell directly through the P2X receptor ion channels (Benham & Tsien, 1987) and in addition the membrane depolarisation associated with P2X receptor activation opens L-type voltage gated calcium channels (Lagaud *et al*., 1996). Therefore it was surprising that TNP-ATP was a weak non-selective antagonist of P2X receptor mediated contractions in whole tissue studies. The difference in antagonist potency between whole tissue and isolated cell experiments is likely to be due to the metabolic breakdown of TNP-ATP in whole tissue preparations. Previous studies have shown the effective concentration of purines can be reduced by ectonucleotidases in whole tissue preparations (Trezise *et al*., 1994; Crack *et al*., 1995). Studies on recombinant P2X receptors have shown that the phosphate group(s) are necessary for the antagonist actions of TNP derivatives as TNP-adenosine is ineffective as an antagonist. Therefore degradation of TNP-ATP, TNP-ADP and TNP-AMP to TNP-adenosine by ectonucleotidases could account for their lack of potency in whole tissue studies.

The potency of TNP-ATP as an antagonist of P2X receptor mediated currents in arterial smooth muscle is similar to that for recombinant $P2X_1$, $P2X_3$ and $P2X_{2/3}$ receptors (Virginio *et al*., 1998). A role for $P2X_3$ or $P2X_{2/3}$ receptors in determining the native arterial smooth muscle phenotype can be discounted based on the restricted tissue distribution of $P2X_3$ receptor subunits to sensory nerves and the agonist actions of 1-β,γ-methylene ATP. 1-β,γ-methylene ATP is an agonist at mesenteric artery P2X receptors (Gitterman & Evans, unpublished observations) and $P2X_1$ receptors (Evans *et al*., 1995) but is ineffective as an agonist at $P2X_3$ receptors (Lewis, 1997) or nodose ganglia P2X receptors (Trezise *et al*., 1995), which are thought to result from the expression of $P2X_{2/3}$ receptors (Lewis *et al*., 1995). These observations provide further evidence that arterial P2X receptors correspond to the $P2X_1$ receptor subtype.

In whole tissue experiments the potency of TNP-ATP (IC_{50} in the μM range) is similar to that reported for recombinant $P2X_2$, $P2X_4$ and $P2X_7$ receptors (Virginio *et al*., 1998). These recombinant phenotypes cannot account for the native P2X receptor phenotype in the mesenteric artery as they are insensitive to α,β-meATP and are expressed at levels below detection by *in situ* hybridisation in smooth muscle preparations (Collo *et al*., 1996). Therefore, if contractions to α,β-meATP are mediated by distinct non- $P2X_1$-like receptors, which is considered unlikely (see above), their properties cannot be accounted for by the complement of P2X receptors currently identified at the molecular level.

In summary TNP-ATP is a highly potent antagonist (IC_{50} ~ 2 nM) of P2X receptor mediated inward currents in rat mesenteric artery smooth muscle cells. These observations are consistent with other evidence to suggest that arterial smooth muscle P2X receptors result from the expression $P2X_1$ receptor subunits. However much higher concentrations of TNP-ATP (~ 30 μM) are required to inhibit P2X receptor mediated constrictions of mesenteric artery rings. This shows that based solely on contraction studies the erroneous conclusion that $P2X_1$-like receptors are not involved in mediating the response to α,β-meATP could be made. The difference in potency probably results from the metabolic breakdown of TNP-ATP in whole tissue studies. Thus the synthesis of metabolically

stable TNP-ATP analogues for example α,β-methylene TNP-ATP may yield highly potent subtype selective P2X receptor antagonists for future whole tissue and *in vivo* studies.

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