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The effect of PPADS as an antagonist of inositol (1,4,5)trisphosphate induced intracellular calcium mobilization

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1 Brain capillary endothelial cells responded to uridine 5'-triphosphate (UTP) and adenosine 5'-triphosphate (ATP) by activation of phospholipase C and by large changes in $[Ca^{2+}]_i$. These cells expressed mRNA sequences identical to the sequence of the P_{2Y2}-purinoceptor of rat pituitaries.

2 Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) at 100 μM did not prevent UTP and ATP induced accumulations of total [^{3}H]-inositol (poly)phosphates. It inhibited UTP and ATP induced intracellular Ca^{2+} mobilization ($IC_{50}=30$ μM) by non competitive mechanism.

3 PPADS (100 μM) inhibited endothelin-1 induced accumulation of total [^{3}H]-inositol (poly)phosphates by less than 20% and prevented most of endothelin-1 induced intracellular Ca^{2+} mobilization ($IC_{50}=30$ μM).

4 PPADS (100 μM) had no action on ionomycin induced intracellular Ca^{2+} mobilization.

5 Microinjection of inositol (1,4,5)trisphosphate ($InsP_3$) into *Xenopus* oocytes induced large Ca^{2+} activated Cl^- currents that were prevented by heparin and by PPADS.

6 It is concluded that PPADS does not recognize rat P_{2Y2}-purinoceptors and prevents UTP and ATP induced intracellular Ca^{2+} mobilization by a non-specific mechanism that could involve the inhibition of $InsP_3$ channels.

Keywords: P_{2Y2}-purinoceptors; ATP; UTP; inositol (1,4,5)trisphosphate ($InsP_3$) channels

Introduction

Extracellular nucleotides elicit diverse biological responses in almost all the tissues and cell types that have been studied. They recognize two classes of receptors. P_{2X}-purinoceptors are ligand-gated channels which are permeable to Ca^{2+} and other cations. P_{2Y}-purinoceptors are metabotropic receptors that are coupled to phospholipase C and induce mobilization of intracellular Ca^{2+} stores via the production of inositol (1,4,5)trisphosphate ($InsP_3$) (Abbracchio & Burnstock, 1994; Dubyak & El-Moatassim, 1993; Fredholm *et al.*, 1994).

Further progress in the study of purinoceptors is hampered by the lack of potent and selective antagonists. Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) has been shown to act as a potent antagonist of P_{2X}-purinoceptors (Lambrecht *et al.*, 1992; Ziganshin *et al.*, 1993). It was also shown to be an antagonist of P_{2Y}-purinoceptors, albeit with a lower potency. In addition, PPADS has been suggested to be a useful tool to distinguish between P_{2Y}- and P_{2U}-purinoceptor-mediated responses. In bovine aortic endothelial cells, PPADS inhibits the action of selective agonists of P_{2Y}-purinoceptors (adenosine 5'-diphosphate (ADP) and 2-methylthio ATP) on phospholipase C activity; it did not inhibit the actions of agonists of P_{2U}-purinoceptors (Brown *et al.*, 1995). However, different results have been obtained in astrocytes from the dorsal spinal cord of the rat (Ho *et al.*, 1995). In these cells, PPADS inhibited uridine 5'-triphosphate (UTP) as well as 2-methylthio ATP induced intracellular Ca^{2+} mobilization. This led to the suggestion that astrocytes expressed a UTP receptor distinct from the P_{2U}-purinoceptor of aortic endothelial cells (Ho *et al.*, 1995).

Previous pharmacological evidence indicated that brain capillary endothelial cells express a phospholipase C coupled P_{2U}-purinoceptor that recognizes ATP and UTP (Frelin *et al.*, 1993; Vigne *et al.*, 1994). In this paper we define the actions of PPADS on UTP and ATP responses. The results showed that PPADS blocks UTP and ATP induced intracellular Ca^{2+} mobilizations mediated by P_{2Y2}-purinoceptors by a non-specific mechanism that probably involves the inhibition of $InsP_3$ channels.

Methods

Rat brain capillary endothelial cells of the B7 clone were grown as previously described (Vigne *et al.*, 1989). The culture medium was Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

For intracellular Ca^{2+} measurements, cells were loaded with 5 μM indo-1/AM for 2 h in complete culture medium at 37°C. After dissociation from the culture dishes, cells were centrifuged at low speed and resuspended into an Earle's salt solution (composition, mM: NaCl 140, KCl 5, $CaCl_2$ 1.8, $MgSO_4$ 0.8, glucose 5, HEPES 25, pH 7.4). Flow cytometric analysis of the indo-1 fluorescence was performed as previously described (Feolde *et al.*, 1995) by use of a FacStar Plus (Becton-Dickinson). To obtain dose-response curves for agonists and PPADS, cells were mixed with the desired concentrations of PPADS for 15 min and then the agonists. The mean indo-1 fluorescence ratio of 1000 cells was measured at 15 s which corresponded to the peak of the response (Vigne *et al.*, 1990; Frelin *et al.*, 1993). The acquisition time was 2 s.

For phospholipase C measurements, cells grown to confluency into six well plates were labelled to equilibrium with 2 μCi ml^{-1} *myo*-[2-^{3}H]-inositol (19 Ci $mmol^{-1}$, Amersham) in complete culture medium. After being washed with an Earle's salt solution, cells were incubated for 15 min at 37°C

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in a 100 mM NaCl, 40 mM LiCl modified Earle's solution in the absence or the presence of PPADS and then exposed to agonists. After 5 min, the radioactivity incorporated into total inositol (poly)phosphates was determined as previously described (Feolde *et al.*, 1995).

Devitellinized *Xenopus* oocytes were prepared as previously described (Guillemare *et al.*, 1992). Whole oocyte currents were recorded at −70 mV by the conventional two microelectrode voltage clamp technique. Oocytes were continually superfused with a physiological bath solution (composition, mM: NaCl 140, $CaCl_2$ 1.8, $MgCl_2$ 2, HEPES 5, pH 7.4) at room temperature. Electrodes contained 3 M KCl and had resistances from 0.2 to 1 MΩ. Following electrode penetration, $InsP_3$ was microinjected with PPADS or heparin by applying pressure pulses to a glass micropipette (Parker & Ivorra, 1991) and the current signal was recorded by a voltage clamp amplifier.

Endothelin-1 was from Neosystems. PPADS was from Research Biochemicals International.

Means ± s.e.mean are shown. When no error bar is presented in the figures, it was smaller than the size of the points. Dose-response curves were fitted to a logistic function by use of the SigmaPlot software.

Results

Brain capillary endothelial cells responded to ATP and UTP by activations of phospholipase C and large changes in $[Ca^{2+}]_i$ that were attributed to P_{2U}-purinoceptors (Frelin *et al.*, 1993; Vigne *et al.*, 1994). Partial sequence data obtained after PCR amplification of reverse transcribed mRNAs indicated the expression of messages that were highly homologous to human and murine P_{2Y2}-purinoceptors (Feolde *et al.*, 1995). The sequence of the PCR product was fully identical to the P_{2Y2}-purinoceptor sequence recently obtained from rat pituitaries (GenBank accession number L 46685).

Addition of ATP and UTP to B7 cells induced an activation of phospholipase C and large increases in cytosolic Ca^{2+} concentration (Frelin *et al.*, 1993). Figure 1 shows dose-response curves for ATP- and UTP-induced formation of inositol (poly)phosphates. This formation was not modified by 100 μM PPADS.

Different results were obtained when ATP and UTP induced increases in cytosolic Ca^{2+} were analysed. We first observed that addition of 100 μM PPADS at the same time as ATP or UTP did not modify agonist-induced changes in $[Ca^{2+}]_i$. Yet when cells were exposed to PPADS for 10 to

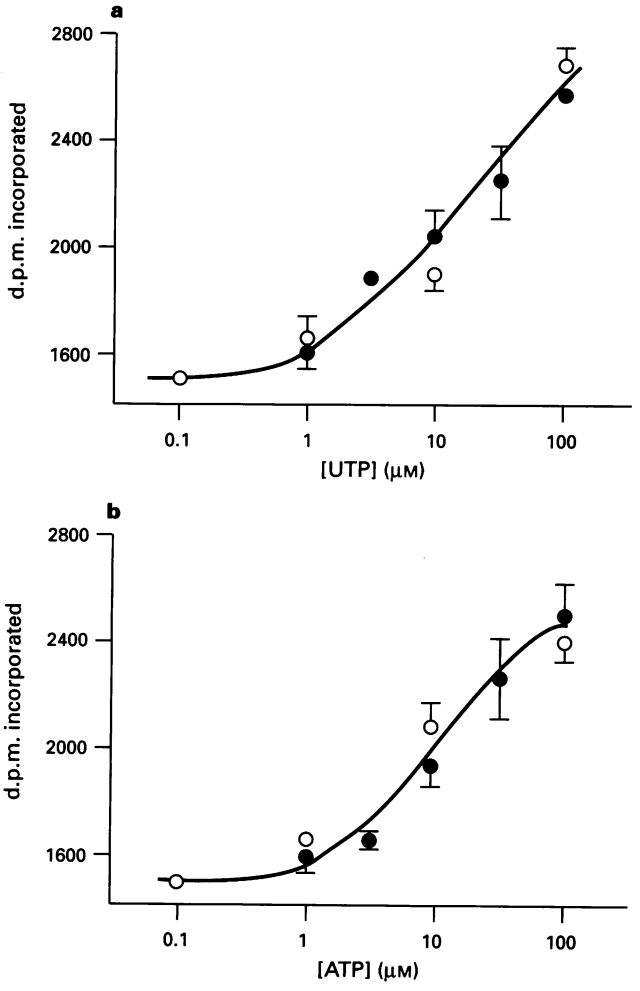

Figure 1 Dose-response curves for UTP (a) and ATP (b) induced formation of total inositol (poly)phosphates. Experiments were performed in the absence (○) or the presence (●) of 100 μM PPADS. Cells that had been labelled to equilibrium with [^{3}H]-inositol were exposed for 15 min to 100 μM PPADs and then to the indicated concentrations of ATP or UTP. The production of total inositol (poly)phosphates was measured after 5 min. Means ± s.e.-mean (vertical lines) of triplicate determinations are indicated. In two other experiments, 100 μM PPADS was found not to alter ATP or UTP (0.1 mM) responses.

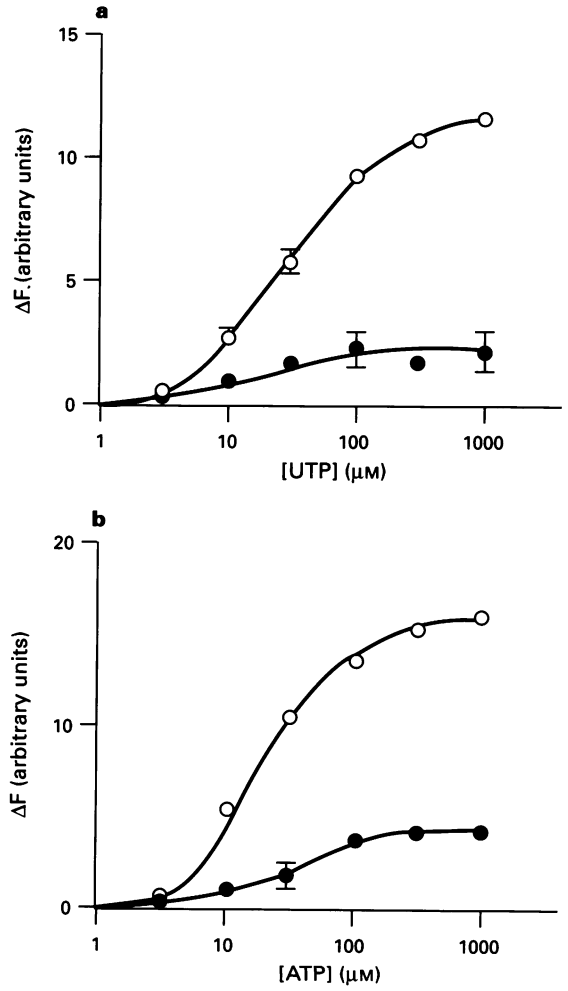

Figure 2 Dose-response curves for the actions of UTP- (a) and ATP- (b) induced changes in the mean indo-1 fluorescence ratio (used to measure intracellular Ca^{2+} mobilization). Experiments were performed in the absence (○) or the presence of 50 μM PPADS (●). Cells were exposed to PPADS for 15 min, challenged with the indicated concentrations of UTP or ATP and mean indo-1 fluorescence ratios were determined after 15 s. Means ± s.e.mean (vertical lines) of triplicate measurements are indicated. Identical results were obtained in 2 other experiments.

15 min before the addition of agonists, PPADS decreased ATP and UTP responses to a large extent. The half-time for the development of PPADS action was about 2 min. Figure 2a presents dose-response curves for UTP induced increase in cytosolic Ca^{2+} measured in the absence or the presence of 50 μM PPADS. The main effect of PPDAS was to decrease the maximum efficacy of UTP. EC_{50} values for the action of UTP were not modified by PPADS treatment. They were 33 ± 3 μM and 12 ± 5 μM in the absence and the presence of PPADS, respectively. This indicated a non-competitive type of inhibition. Similarly, PPADS inhibited the effect of ATP on $[Ca^{2+}]_i$ in a non-competitive manner (Figure 2b). EC_{50} values for the effect of ATP were 20 ± 4 μM and 32 ± 7 μM in the absence and the presence of 50 μM PPADS, respectively. Figure 3 shows dose-response curves for PPADS inhibition of the effects of ATP and UTP on $[Ca^{2+}]_i$. The IC_{50} value for PPADS inhibition of the effect of UTP (0.1 mM) was 32 ± 3 μM. The corresponding value for ATP (0.1 mM) was 35 ± 4 μM.

Taken together these results suggested that PPADS did not prevent P_{2Y2}-purinoceptor activation, measured as the production of inositol (poly)phosphates, but that it acted downstream of phospholipase C. Further evidence for this hypothesis was obtained in experiments with endothelin-1 (ET-1). ET-1 is a potent agonist of phospholipase C in brain capillary endothelial cells (Vigne *et al.*, 1990). It acts via BQ-123 sensitive ETA receptors (Vigne *et al.*, 1993) Figure 4a shows that 100 μM PPADS inhibited ET-1 induced formation of inositol (poly)phosphates by less than 20%. At the same concentration, PPADS almost completely prevented ET-1 induced increases in cytosolic Ca^{2+} immobilization (Figure 4b). The observed IC_{50} value was 27 ± 5 μM, close to the values observed in experiments in which ATP and UTP were used as agonists.

One possibility for these results could be that preincubation of the cells with PPADS induced a depletion of intracellular Ca^{2+} stores and prevented the generation of $InsP_3$ by phospholipase C which activates $InsP_3$ channels. To test this hypothesis, we analysed the action of PPADS on the ionomycin-induced rise in $[Ca^{2+}]_i$. Experiments were performed in the presence of 4 mM EGTA to prevent Ca^{2+} influx. Figure 5 shows that PPADS did not modify ionomycin (1 μM)-induced intracellular Ca^{2+} mobilization.

Another possibility for the action of PPADS could be that it enters the cells and inhibits $InsP_3$ receptors. To test this hypothesis we used *Xenopus* oocytes. Figure 6a shows that injection of 1 μM $InsP_3$ into oocytes induced a large Ca^{2+}-activated Cl^- current that was prevented by 10 μM heparin. Likewise, PPADS (0.1 mM) partially prevented $InsP_3$-induced activation of Cl^- currents. This action was not due to the blockade of Cl^- channels as an injection of Ca^{2+} into PPADS-treated oocytes still induced a large outward Cl^- current (data not shown). Figure 6b presents a summary of the results that were obtained. It shows a dose-dependent action of injected $InsP_3$ on Cl^- currents and an inhibitory action of PPADS at all concentrations of $InsP_3$ tested. At 100 μM, PPADS almost completely blocked $InsP_3$ (0.1 μM) induced Cl^- currents.

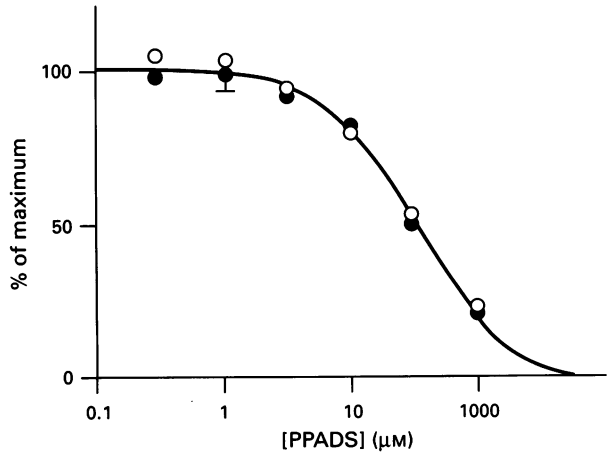

Figure 3 Dose response curve for PPADS inhibition of UTP- or ATP-induced intracellular Ca^{2+} mobilization. Cells were exposed to the indicated concentrations of PPADS for 15 min and then to 0.1 mM UTP (●) or 0.1 mM ATP (○). Mean indo-1 fluorescence ratios were determined after 15 s. Means ± s.e.mean (vertical lines) of triplicate measures are indicated. Identical results were obtained in 2 other experiments.

Discussion

This paper shows an inhibitory action of PPADS on UTP and ATP responses mediated by P_{2Y2}-purinoceptors in brain capillary endothelial cells. This action was not related to P_{2Y2}-purinoceptor inhibition for the following reasons: (i) inhibition by PPADS of UTP and ATP induced changes in $[Ca^{2+}]_i$ was non-competitive (Figure 2), (ii) PPADS also inhibited ET-1 induced intracellular Ca^{2+} mobilization (Figure 4) and (iii) PPADS had no action on UTP or ATP induced activations of

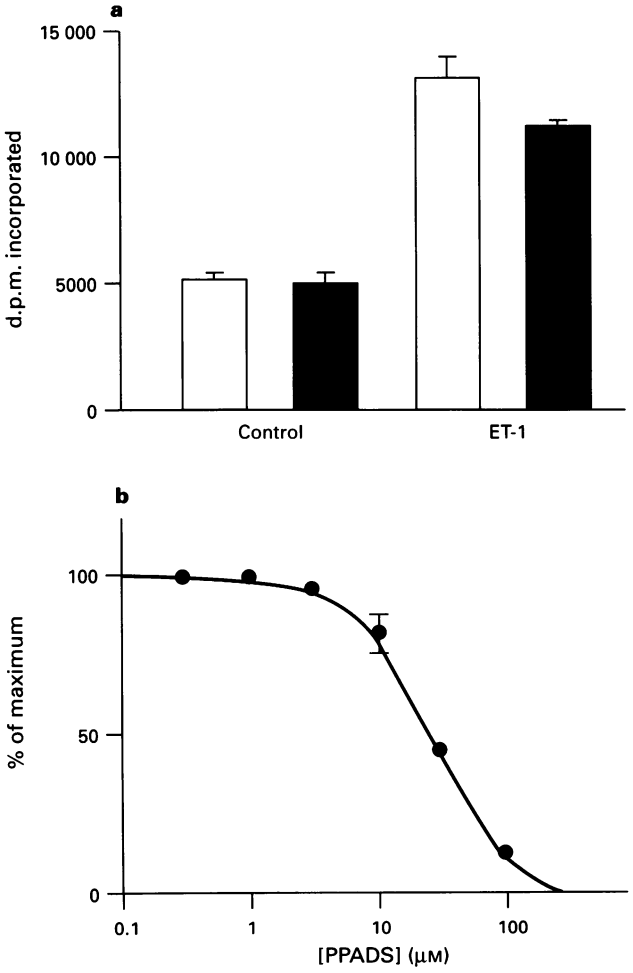

Figure 4 The effect of PPADS on endothelin-1 (ET-1) signalling. (a) Cells that had been labelled to equilibrium with [^{3}H]-inositol were exposed for 15 min to 100 μM PPADS (solid columns) or vehicle (open columns) and then to 100 nM ET-1 or vehicle as indicated. The production of total inositol (poly)phosphates was measured after 5 min. Means ± s.e.mean (vertical lines) of triplicate measurements are indicated. An identical result was obtained in another experiment. (b) Dose-response curve for PPADS inhibition of ET-1 induced intracellular Ca^{2+} mobilization. Cells were exposed to the indicated concentrations of PPADS for 15 min and then to 100 nM ET-1. Mean indo-1 fluorescence ratios were determined after 15 s. Means ± s.e.-mean (vertical lines) of triplicate measurements are indicated. Similar results were obtained in another experiment.

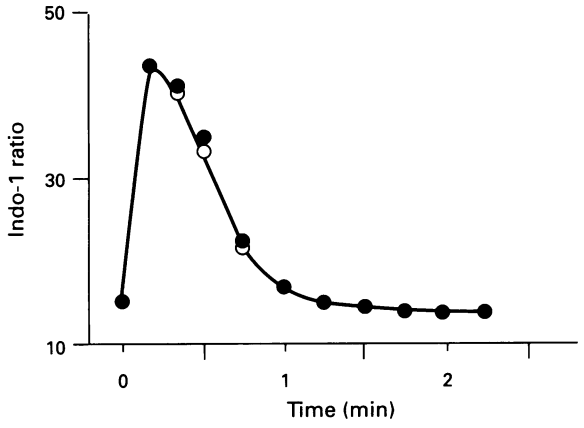

Figure 5 The lack of an effect of PPADS on ionomycin induced intracellular Ca^{2+} mobilization. Cells were exposed to 100 μM PPADS (●) or vehicle (○) for 15 min. EGTA (4 mM) was then added to chelate extracellular Ca^{2+} and cells were treated with 1 μM ionomycin. Mean indo-1 fluorescence ratios were collected at 10 s intervals. Means of three experiments are shown; s.e.mean was smaller than the size of the symbols.

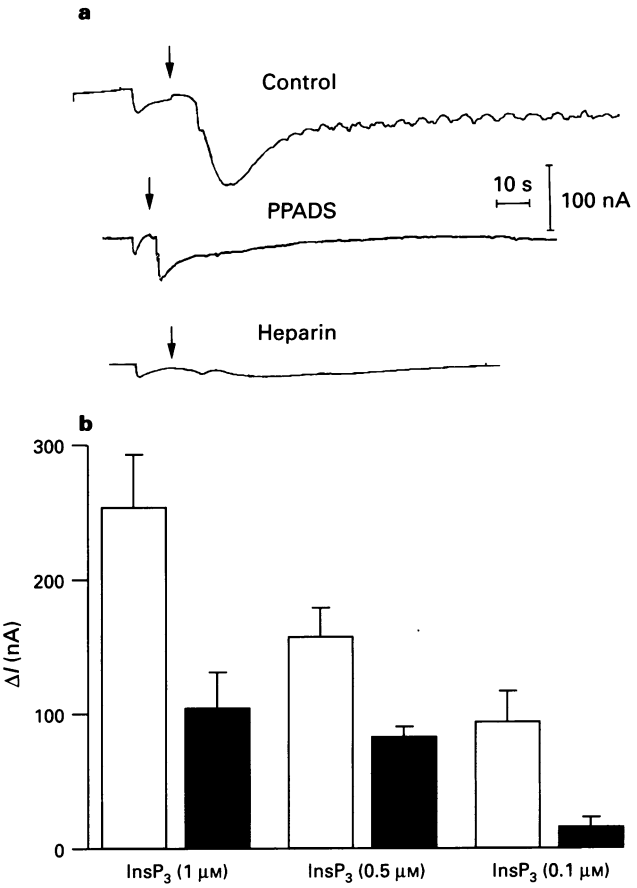

Figure 6 The effect of microinjected PPADS on $InsP_3$-induced Cl^- currents in *Xenopus* oocytes. (a) Typical traces showing the action of 1 μM $InsP_3$ on inward Cl^- currents in control oocytes and oocytes that had been microinjected with 10 μM heparin or with 100 μM PPADS. Oocytes were maintained at −70 mV. (b) Dose-response curves for $InsP_3$ induced Cl^- currents in the absence (open columns) and presence (solid columns) of 100 μM PPADS. Means ± s.e.mean (n = 3–4) are indicated. Each injection had a volume of 10 nl. Concentrations were calculated on the assumption of a mean oocyte volume of 1 μl.

phospholipase C (Figure 1). Knowing that PPADS did not deplete intracellular Ca^{2+} stores, as evidenced by experiments with ionomycin (Figure 5), an obvious hypothesis could be that PPADS entered cells and inhibited $InsP_3$ induced intracellular Ca^{2+} mobilization. PPADS uptake by endothelial cells could not be documented but experiments with *Xenopus* oocytes clearly indicated that microinjected PPADS inhibited $InsP_3$ induced intracellular Ca^{2+} mobilization and the resulting activation of Ca^{2+}-dependent Cl^- channels.

Earlier work has suggested that PPADS can be used as an antagonist of P_{2X}-purinoceptors (Lambrecht *et al.*, 1992; Ziganshin *et al.*, 1993) and of P_{2Y1}-purinoceptors (Brown *et al.*, 1995). Its interaction with P_{2U}-purinoceptors is less clear. In aortic endothelial cells, PPADS did not inhibit UTP-induced activation of phospholipase C and it was suggested to be a selective antagonist of P_{2Y1}-purinoceptor responses (Brown *et al.*, 1995). In astrocytes from the dorsal spinal cord of the rat, PPADS inhibited UTP-induced changes in $[Ca^{2+}]_i$ (Ho *et al.*, 1995). This type of observation has led to the suggestion that there may be PPADS-sensitive and PPADS-insensitive P_{2U}-purinoceptors (Ho *et al.*, 1995). This possibility was not substantiated by molecular data. The results presented in this article show that high concentrations of PPADS inhibit P_{2Y2}-purinoceptor-mediated intracellular Ca^{2+} mobilization but not P_{2Y2}-purinoceptor-mediated activation of phospholipase C. They indicate that large (> 10 μM) concentrations of PPADS should be used with caution for assessing P_2-purinoceptor responses. A 10 μM concentration of PPADS is sufficient to block most of the P_{2X} and P_{2Y1}-purinoceptor-mediated responses (Lambrecht *et al.*, 1992; Ziganshin *et al.*, 1993; Brown *et al.*, 1995). At 100 μM, PPADS affects procedures monitoring Ca^{2+} mobilization or downstream of it. The same concentrations of PPADS do not affect phospholipase C measurements.

Our results also suggest new uses for PPADS. In *Xenopus* oocytes, microinjected PPADS prevents $InsP_3$ induced intracellular Ca^{2+} mobilization and the resulting activation of Ca^{2+} activated Cl^- channels (Figure 6). PPADS thus appears as a new inhibitor of $InsP_3$-induced mobilization of intracellular Ca^{2+} stores in addition to heparin. It should be noted, however, that the sensitivity of $InsP_3$ channel to PPADS is 50 times less than that to heparin (Guillemette *et al.*, 1989).

PPADS prevents UTP, ATP and ET-1 induced intracellular Ca^{2+} mobilization without affecting much their capacities to activate phospholipase C. Such an action is shared by thapsigargin, an inhibitor of Ca^{2+} ATPases of the sarco-endoplasmic reticulum. However, one difference is that while intracellular Ca^{2+} stores are depleted after thapsigargin treatment, they are full with Ca^{2+} after PPADS treatment. Thus PPADS might also be a useful tool to analyse the regulation of intracellular Ca^{2+} signalling.

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