Endogenous extracellular purine nucleotides redirect α_2-adrenoceptor signaling

Karl E.O. Åkerman*, Johnny Näsman, Per-Eric Lund, Ramin Shariatmadari, Jyrki P. Kukkonen

Department of Physiology, Uppsala University, BMC, P.O. Box 572, S-75123 Uppsala, Sweden

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Abstract Many receptors coupled to inhibitory G_o/G_i-type G proteins often also produce stimulatory signals like Ca^{2+} mobilisation. When expressed in CHO cells the α_2-adrenoceptor subtypes α_{2A}, α_{2B} and α_{2C} mobilised Ca^{2+}. These responses were strongly reduced by the P_{2Y}-purinoceptor antagonist suramin. A large proportion of the total pool of purine nucleotides was found extracellularly. Removal of extracellular nucleotides with apyrase or by constant perfusion had a similar effect as suramin. These treatments did not affect the α_2-adrenoceptor-mediated inhibition of cAMP production. This indicates that cells may be primed or their signaling pathways redirected towards Ca^{2+} mobilisation by 'autocrine' release of nucleotides.

Key words: α_2-adrenoceptor; P_{2Y}-receptor; Apyrase; Suramin; Calcium; Fura-2

1. Introduction

A major group of G protein-coupled receptors transduce signals through G_o/G_i-proteins. These receptors couple to ion channels and to inhibition of cAMP production, and are considered mainly inhibitory in their action. However, in some cells a stimulatory response such as Ca^{2+} elevation is observed upon activation of this type of receptors [1]. This has in many studies been observed for α_2-adrenoceptors. The α_2-adrenoceptors couple in many cells to production of inositol-1,4,5-trisphosphate [2–5] and Ca^{2+} mobilisation [3–8] by a mechanism which is sensitive to inhibitors of phospholipase C such as U73122 [4,5]. The Ca^{2+} elevation is pertussis toxin-sensitive [3–8] and hence due to G_o/G_i-protein activation. Unlike α_q, the α subunits of G_o/G_i (α_o/α_i) are unable to activate phospholipase C-β [9,10] but $\beta\gamma$ subunits may activate the enzyme [11–13]. Indeed, evidence for the involvement of $\beta\gamma$ subunits in α_2-receptor-mediated Ca^{2+} mobilisation has been presented [5]. The stimulatory activity of α_2-receptors is of physiological importance for mediation of functions such as platelet aggregation, secretion and contraction of smooth muscle [14]. In all these cases, Ca^{2+} elevation is either directly or indirectly implicated. Other G_o/G_i-coupled receptors, such as opiate receptors, have been able to elevate cytosolic Ca^{2+} only after prior stimulation of G_q-coupled receptors such as muscarinic receptors [15]. Similarly, activation of G_q-coupled P_{2Y}-purinoceptors by addition of ATP has been shown to amplify pertussis toxin-sensitive Ca^{2+} mobilisation through A_1 adenosine [16] and M_4 muscarinic receptors [17]. Most cells express P_{2Y}-purinoceptors which may be activated already at the physiological extracellular levels of purine nucleotides [18,19]. High extracellular levels of purine nucleotides are usually a result of exocytotic or anion transporter-mediated release [18,19]. Endogenously activated P_{2Y}-receptors may thus enable G_o/G_i-coupled receptors to mobilise Ca^{2+}.

The aim of this study was to use the fura-2 method [20] to determine whether released endogenous purine nucleotides acting on endogenous P_{2Y}-receptors cooperate to enable α_2-adrenoceptors to mobilise intracellular free Ca^{2+}.

2. Materials and methods

2.1. Reagents

Apyrase, EGTA (ethylene glycol-bis[β-aminoethyl ether]N,N,N′,N′-tetraacetic acid), noradrenaline, probenecid (*p*-[dipropylsulfamoyl]-benzoic acid) and suramin were purchased from Sigma Chemical Co. (St. Louis, MO). Digitonin was purchased from Merck AG (Darmstadt, Germany) and fura-2 acetoxymethyl ester from Molecular Probes Inc. (Eugene).

2.2. Cells

CHO (Chinese hamster ovary) cells (American Type Culture Collection, Manossas, VI, USA), transfected as described in [21] were grown in MEM alpha (Gibco, Paisley, UK) supplemented with 0.22% (w/v) $NaHCO_3$, 100 U/ml penicillin G, 80 U/ml streptomycin (Sigma) and 5% (v/v) foetal calf serum (Gibco) at 37°C in 5% CO_2 in an air ventilated humidified incubator in 260 ml plastic culture flasks (75 cm^2 bottom area; Nunc A/S, Roskilde, Denmark) or in plastic culture dishes (diameter 94 mm; Greiner GmbH, Frickenhausen, Germany).

2.3. Ca^{2+} measurements

The fluorescent Ca^{2+} indicator fura-2 [20] was used to monitor changes in intracellular Ca^{2+}. The cells were harvested using phosphate buffered saline containing 0.2 g/l ethylenediaminetetraacetic acid (EDTA), spun down, and loaded at 37°C in MEM alpha supplemented with 0.002% (w/v) bovine serum albumin, 1 mM probenecid and 4 μM fura-2 acetoxymethyl ester for 20 min. The cells were washed once with Ca^{2+}-free TBM and stored on ice as pellets (medium removed). The measurement of intracellular free calcium was carried out as follows: one pellet was resuspended in TBM (TES buffered medium consisting of 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$ and 20 mM 2-([2-hydroxy-1,1-bis{hydroxymethyl}-ethyl]amino) ethane sulphonic acid (TES) adjusted to pH 7.4 with NaOH) at 37°C and placed in a stirred quartz microcuvette in a thermostated cell-holder within a fluorescence spectrophotometer. Fluorescence was monitored either with a Hitachi F-2000 or a Hitachi F-4000 fluorescence spectrophotometer at the wavelengths 340 nm (excitation), 505 nm (emission) or with a PTI QuantaMaster fluorescence spectrophotometer at the wavelengths 340/360/380 nm (excitation), 505 nm (emission). Suramin or apyrase were added 4 min prior to the addition or noradrenaline. The experiments were calibrated using 60 μg/ml digitonin, which gives the maximum value of fluorescence (F_{max}) and 10 mM EGTA, which gives the minimum value of fluorescence (F_{min}). The free Ca^{2+} concentration was calculated from the fluorescence (F) using the equation:

*Corresponding author. Fax: +46 (18) 4714938.
E-mail: karl.akerman@fysiologi.uu.se

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$$[Ca^{2+}] = (F-F_{min})/(F_{max}-F)\times 224\ nM$$

in which the extracellular fura-2 fluorescence is subtracted from the F values.

The Ca^{2+} imaging experiments were performed and the data analysed using Intracellular Imaging InCyt2 fluorescence imaging system (Cincinnati, OH, USA). The cells were kept in TBM at 37°C and excited by alternating wavelengths of 340 and 380 nm using narrow band excitation filters and the fluorescence was measured through a 430 nm dichroic mirror and a 510 nm barrier filter with a Cohu CCD camera. One ratioed image was acquired per second.

2.4. Measurements of extracellular purine nucleotides and cAMP

The CHO cells on petri dishes were incubated with 5 μCi/ml [^{3}H]adenine in culture medium at 37°C for 2 h before they were harvested as above and spun down. The pellet was resuspended in TBM and divided into aliquots of about 10^6 cells/0.8 ml. The cells were preincubated with 0.5 mM IBMX (3-isobutyl-1-methyl-xanthine) and 150 μM quinacrine for 10 min at 37°C with or without 2 U/ml apyrase. After another 10 min incubation with the drugs the cells were spun down and the supernatants removed. Supernatants from control and apyrase treated samples were loaded into Dowex columns and [^{3}H]ATP eluted with 2 ml H_2O. The eluted radioactivity was determined in a liquid scintillation counter. The pellets were resuspended in 1 ml 0.33 M perchloric acid containing about 1500 cpm [^{14}C]cAMP. Cyclic AMP was isolated by sequential Dowex/alumina ion exchange chromatography and radioactivity was determined in a liquid scintillation counter [22]. The conversion of [^{3}H]ATP to [^{3}H]cAMP was calculated as a percentage of total cellular [^{3}H]ATP and normalised to the recovery of [^{14}C]cAMP.

3. Results

When added to CHO cells expressing α_2-adrenoceptors, ATP (10 μM) caused a rapid slowly declining increase in intracellular free Ca^{2+} (Fig. 1A). Pretreatment with EGTA did not affect the magnitude of the response to ATP. In control conditions the peak $\Delta[Ca^{2+}]_i$ response to 10 μM ATP was 120 ± 4 nM (± S.D., n = 4) and in the presence of 2 mM EGTA it was 130 ± 8 nM (± S.D., n = 4). This suggests that the Ca^{2+} signals are due to intracellular Ca^{2+} mobilisation and hence result from P_{2Y}-receptor activation. When measurements were performed in the presence of 2 U/ml apyrase (an enzyme that hydrolyses adenine nucleotides to adenosine and phosphate) the response to ATP became more transient indicating that the added ATP was rapidly hydrolyzed (Fig. 1B). Addition of 100 μM suramin – a relatively subtype non-selec-

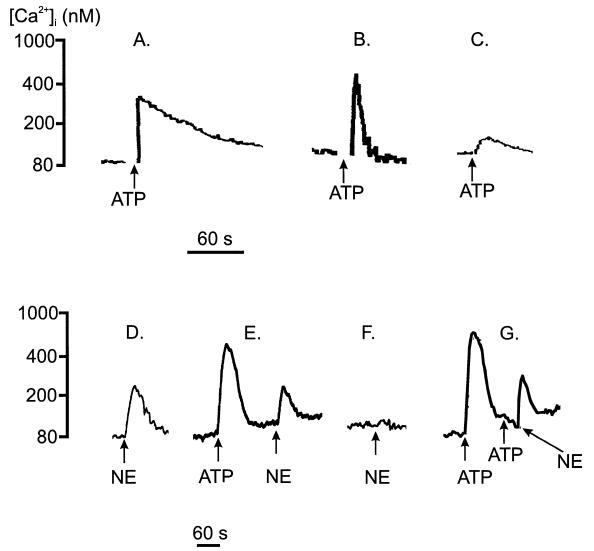

Fig. 1. The effect of apyrase on ATP and noradrenaline-mediated increase in intracellular free Ca^{2+} as determined using fura-2. CHO cells expressing the α_{2C} subtype were detached from the culture dishes using EDTA and loaded with fura-2 acetoxymethyl ester (A–G). Original fluorescence recordings from cell suspensions (about 500 000 cells/ml) are shown. In A, 10 μM ATP was added where indicated. In B and C, 10 μM ATP was added to cells pretreated for 4 min with either 2 U/ml apyrase or 100 μM suramin, respectively. In D, 10 μM noradrenaline, and in E, 1 mM ATP and 10 μM noradrenaline were added. In F and G, cells were pretreated for 4 min with apyrase whereafter 10 μM noradrenaline or 1 mM ATP and 10 μM noradrenaline, respectively, were added where indicated.

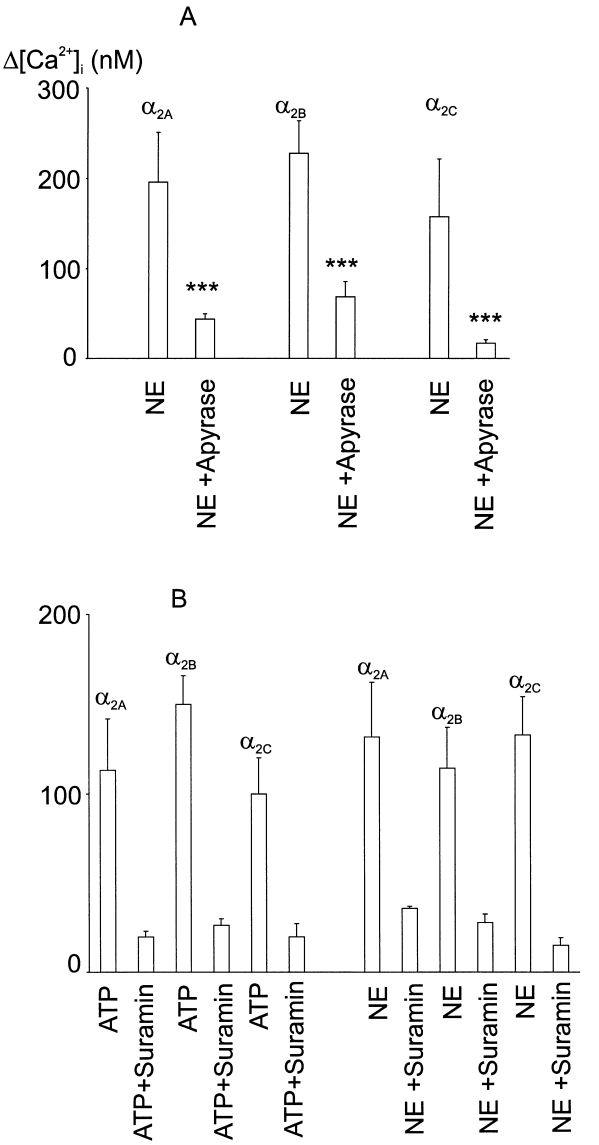

Fig. 2. Effect of apyrase and suramin on ATP and noradrenaline-mediated increase in intracellular free Ca^{2+}. In A, the peak values for the increase in fura-2 fluorescence from several measurements in the presence and absence of apyrase, similar to those in Fig. 1D and F, have been averaged (± S.D.), for the different α_2-adrenoreceptor subtypes (α_{2A}, n = 12; α_{2B}, n = 16; α_{2C}, n = 24). In B, experiments were performed as in Fig. 1A and C. Cells were pretreated for 4 min in the absence or in the presence of 200 μM suramin whereafter 10 μM noradrenaline or 10 μM ATP was added. Each data point is an average from 6–8 measurements (± S.D.). The significances are indicated with respect to the untreated controls (***: $P > 0.001$, student's two-tailed t-test).

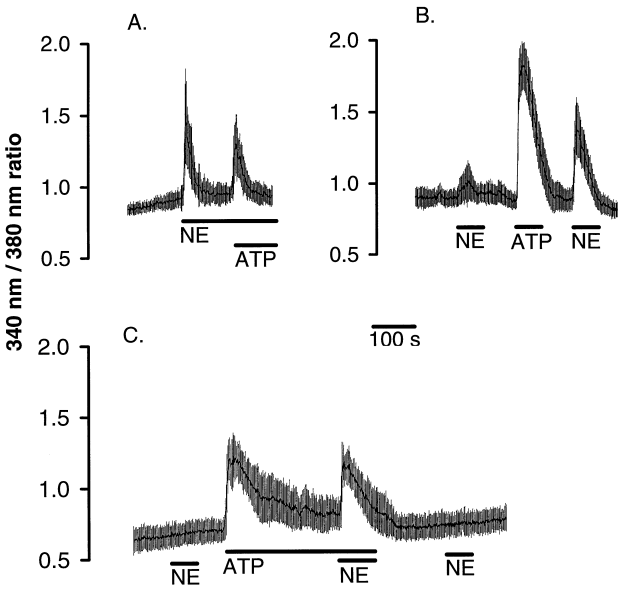

Fig. 3. Effect of perfusion of cells on noradrenaline-mediated increase in intracellular free Ca^{2+}. Single cells were investigated using image analysis. For these experiments the cells were grown on circular glass coverslips (diameter 22 mm) and loaded with 4 μM fura-2 acetoxymethyl ester at 37°C in TBM for 20 min. In A, cells were placed in a chamber with 1 ml of TBM and preincubated for 5 min. Noradrenaline and ATP were added by application directly into the chamber. A volume of 0.35 ml was removed from the top of the cells, mixed with the ligands and readded into the chamber. The data are shown as an average of 16 cells (±S.D.). Four similar experiments were performed with qualitatively similar results. In B, the coverslips were placed in a perfusion chamber (0.2 ml volume) and perfused with a rate of 0.5 ml per min for 5 min before applications were made. Where indicated, noradrenaline (10 μM) or ATP (10 μM) was applied by perfusion. The data are shown as an average of 31 cells (±S.D.). Six similar experiments were performed with qualitatively similar results. In C, conditions were as in B, except that noradrenaline was applied before, during and after a perfusion with 1 μM ATP. The data are shown as an average of 16 cells (±S.D.). Five similar experiments were performed with qualitatively similar results.

tive P_{2Y}-receptor antagonist – significantly reduced the response to ATP (Fig. 1C). The response to 100 nM thapsigargin was unaffected by suramin (in the absence of suramin the $\Delta[Ca^{2+}]_i$ was 36±6 nM, ±S.D., $n=3$ and in the presence of 200 μM suramin it was 37±6 nM, ±S.D., $n=4$, 5 min after the addition of thapsigargin) indicating that the effect of suramin is not on Ca^{2+} mobilisation itself. This indicates that the responses mediated by the endogenous P_{2Y}-receptors in CHO cells [23] are sensitive to these treatments (apyrase or suramin). Addition of noradrenaline (10 μM) to CHO cells expressing the α_2-adrenoceptor subtypes (α_{2A}, α_{2B}, α_{2C}) also resulted in an increase in intracellular free Ca^{2+} (Fig. 1D) as shown previously for α_{2A} [5]. A similar Ca^{2+} elevation to noradrenaline was seen if ATP (1 mM) was added prior to noradrenaline (Fig. 1E). The Ca^{2+} mobilising effect of noradrenaline was considerably attenuated in cells treated with apyrase to remove extracellular purine nucleotides (Fig. 1F). If ATP was added immediately prior to noradrenaline the response to noradrenaline reappeared (Fig. 1G). The noradrenaline-induced Ca^{2+} response was reduced by 83%, 70% and 95% in α_{2A}-, α_{2B}- and α_{2C}-expressing cells, respectively, by the apyrase treatment (Fig. 2A).

The experiments described above indicate that the α_2-adrenoceptor-mediated Ca^{2+} response requires the presence of purine nucleotides acting on the endogenous P_{2Y}-receptors. To test whether apyrase-sensitive adenine nucleotides were present during the Ca^{2+} measurements cells were labeled with [^{3}H]adenine and the cellular supernatants were assayed for ATP/ADP chromatographically. Of the eluted ATP/ADP fraction [24], 73±2% (209451±5453 cpm; average±S.E.M., $n=3$) was found intracellularly and 27±2% (78923±3692 cpm) extracellularly. Treatment with apyrase did not affect the intracellular activity (201820±6161 cpm) but reduced the extracellular activity to 1.6±0.1% (3374±167 cpm) of the total radioactivity in this fraction. This suggests that a large extracellular pool of one or both of these nucleotides exists and that the endogenous P_{2Y}-receptor ligands ADP and/or ATP were indeed largely removed by the apyrase treatment.

Addition of 200 μM suramin prior to noradrenaline (10 μM) or ATP (10 μM) caused a significant reduction of the Ca^{2+} mobilisation induced by either ligand (Fig. 2B).

To exclude the possibility that the effects of apyrase and suramin on the α_2-adrenoceptor-mediated increase in intracellular free Ca^{2+} are due to a direct inhibition of the α_2-adrenoceptors, their effect on the receptor-mediated inhibition of cAMP accumulation was tested. Addition of forskolin to CHO cells expressing α_{2C}-adrenoceptor caused a 20-fold stimulation of cAMP accumulation. Noradrenaline inhibited this cAMP accumulation by 75±6% (average±S.E.M., $n=4$). Neither apyrase (68±3% inhibition) nor suramin (80±4% inhibition) affected this response.

If purine nucleotides present extracellularly indeed modify the responses to the α_2-adrenoceptors activation they should be removable by constantly perfusing the cells. When noradrenaline was applied to cells without perfusion it caused a considerable Ca^{2+} elevation seen as an increase in the 340/380 fluorescence ratio (Fig. 3A). Noradrenaline was, however, unable to induce Ca^{2+} mobilisation in the perfused cells unless it was applied soon after ATP (Fig. 3B) or during constant perfusion with a low concentration of ATP (1 μM).

4. Discussion

The sensitivity of the α_2-adrenoceptor responses to apyrase, suramin and perfusion indicates that the purinergic receptors somehow potentiate α_2-adrenoceptor-mediated Ca^{2+} responses. As the Ca^{2+} responses through α_2-adrenoceptor are dependent on G_i/G_o and require βγ subunits [5] one likely explanation is that these subunits and α_q (or some other G protein α subunit) activated by the purinergic receptors cooperate in their stimulation of phospholipase C-β. Activation of purified phospholipase C-β by βγ, unlike adenylyl cyclase [25], does not have any apparent additional requirement for α subunits [11–13]. The situation may, however, be different in situ in cells where the involvement of α subunits seems likely: a co-activation of phospholipase C-β by α and βγ subunits has been implicated in transfection and reconstitution experiments [26,27]. Mobilisation of Ca^{2+} by G_o/G_i-coupled receptors has in other studies been shown to be potentiated by activation of G_q-coupled receptors [15–17]. It is therefore likely that the pertussis toxin-sensitive Ca^{2+} responses seen in several types of cells upon stimulation of different G_i/G_o-coupled receptors [1] are due to a cooperative mechanism involving endoge-

nously activated G_q-coupled receptors – possibly purinergic receptors.

The presence of extracellular endogenous purine nucleotides should thus be considered in studies describing signaling pathways in cells or tissues. It is of importance to recognise that any basal, spontaneous or stimulated activity may already be modified by the presence of these nucleotides.

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