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Human breast cancer cell line MDA-MB-231 expresses endogenous A_{2B} adenosine receptors mediating a Ca^{2+} signal

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1 Two human breast cancer cell lines, MCF-7 and MDA-MB-231, were screened for the presence of functionally significant adenosine receptor subtypes.

2 MCF-7 cells did not contain adenosine receptors as judged by the lack of an effect of nonselective agonists on adenylyl cyclase activity or intracellular Ca^{2+} levels. MDA-MB-231 cells showed both a stimulation of adenylyl cyclase and a PLC-dependent increase in intracellular Ca^{2+} in response to nonselective adenosine receptor agonists.

3 Both adenosine-mediated responses in MDA-MB-231 cells were observed with the nonselective agonists 5′-*N*-ethylcarboxamidoadenosine (NECA) and 2-(3-hydroxy-3-phenyl)propyn-1-yladenosine-5′-*N*-ethyluronamide (PHPNECA), but no responses were observed with agonists selective for A_1, A_{2A} or A_3 adenosine receptors. The Ca^{2+} signal was antagonized by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and the nonselective antagonist 9-ethyl-8-furyladenine (ANR 152), but not by A_{2A} or A_3 selective compounds.

4 In radioligand binding with [2-^{3}H](4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol) ([^{3}H]ZM 241385), a specific binding site with a K_D value of 87 nM and a B_{max} value of 1600 fmol mg^{-1} membrane protein was identified in membranes from MDA-MB-231 cells.

5 The pharmacological characteristics provide evidence for the expression of an A_{2B} adenosine receptor in MDA-MB-231 cells, which not only mediates a stimulation of adenylyl cyclase but also couples to a PLC-dependent Ca^{2+} signal, most likely *via* $G_{q/11}$. The A_{2B} receptor in such cancer cells may serve as a target to control cell growth and proliferation.

6 The selective expression of high levels of endogenous A_{2B} receptors coupled to two signaling pathways make MDA-MB-231 cells a suitable model for this human adenosine receptor subtype.
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Abbreviations: ANR 152, 9-ethyl-8-furyladenine; CCPA, 2-chloro-N^6-cyclopentyladenosine; CGS 21680, 2-[*p*-(2-carboxyethyl)-phenylethylamino]adenosine-5′-*N*-ethyluronamide; Cl-IB-MECA, 2-chloro-N^6-3-iodobenzyladenosine-5′-*N*-methyl-uronamide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; MRS 1220, 9-chloro-2-(2-furyl)-5-phenylactylamino [1,2,4]triazolo[1,5-*c*]quinazoline; NECA, adenosine-5′-*N*-ethyluronamide; PENECA, 2-phenylethynyladenosine-5′-*N*-ethyluronamide; PHPNECA, 2-(3-hydroxy-3-phenyl)propyn-1-yladenosine-5′-*N*-ethyluronamide; SCH 58261, 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; U-73122, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; U-73343, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-pyrrolidine-2,5-dione; [^{3}H]ZM 241385, [2-^{3}H](4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol)

Introduction

Adenosine has dual functions as a metabolite and as a regulator of cellular processes in most organs. The regulatory functions of adenosine are mediated *via* four subtypes of G protein-coupled receptors which are distinguished as the A_1, A_{2A}, A_{2B} and A_3 subtypes (Fredholm *et al*., 2001a). Owing to their widespread occurrence, adenosine receptors have been considered to be important players in pathophysiological situations associated with increased adenosine release and, therefore, are potential targets for drug treatment in numerous dieseases. In addition to asthma (Feoktistov *et al*., 1998; Spicuzza *et al*., 2003), cardiovascular diseases (Auchampach & Bolli, 1999; Ganote & Armstrong, 2000; Kitakaze & Hori, 2000) and CNS diseases (Ongini & Fredholm, 1996; Müller, 2000), it was recently also suggested that adenosine receptors may play a role in the regulation of tumor growth (for a review, see Spychala, 2000; Merighi *et al*., 2003). In particular, A_3 receptors have been shown to be present in various tumor cells and are thought to be involved in the control of cell growth and proliferation (Madi *et al*., 2003; 2004; Fishman *et al*., 2004). It was also shown that the A_3/A_1 agonist IB-MECA suppresses human breast cancer cell proliferation through an A_3 receptor-independent mechanism (Lu *et al*., 2003). On the other hand, potential tumor-promoting functions of adenosine were also discussed (Spychala, 2000; Merighi *et al*., 2003).

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The release of adenosine is dependent on the metabolic state of a cell and an increase in energy consumption or hypoxia will lead to an enhanced production of adenosine (Illes *et al*., 2000; Merighi *et al*., 2003). It is reasonable to assume, therefore, that metabolically active tumor cells may be characterized by a pronounced adenosine release (Spychala, 2000; Merighi *et al*., 2003). The local release of adenosine may then regulate the growth and development of these tumor cells in adenosine receptor-dependent and independent ways. Consequently, the presence of defined receptor subtypes will be an important determinant for a specific effect of adenosine on the function of a tumor cell. The knowledge of the expression pattern of different tumor cells is essential, therefore, for the development of potential therapeutic regimens targeting adenosine receptors that may aid in more efficient tumor growth control.

In order to identify adenosine receptor subtypes in an estrogen-positive (MCF-7) and in an estrogen-negative (MDA-MB-231) human breast cancer cell line, we screened these two cell lines for cAMP and Ca^{2+} signals in response to the nonselective adenosine receptor agonists NECA and PHPNECA. In MCF-7 cells, we did not detect any functional responses suggesting that these cells do not express functionally significant levels of adenosine receptors. In MDA-MB-231, however, we identified a cAMP and a Ca^{2+} signal with characteristics compatible with the presence of high levels of an A_{2B} adenosine receptor.

Methods

Cell culture

MDA-MB-231 and MCF-7 breast cancer cell lines were grown adherently and maintained in DMEM containing 10% fetal calf serum (FCS), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) at 37°C in 5% CO_2/95% air. Chinese hamster ovary (CHO) cells stably transfected with human A_{2B} receptors in DMEM/F12 without nucleosides, containing 10% FCS, penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), L-glutamine (2 mM) at 37°C in 5% CO_2/95% air and HEK-293 cells stably transfected with human A_{2B} receptors in DMEM containing 10% FCS, penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), L-glutamine (2 mM) at 37°C in 7% CO_2. Cells were split two or three times weekly at a ratio between 1:4 and 1:8. For calcium assays the culture medium was removed, cells were washed with PBS, trypsinized and replated to grow on coverslips precoated with polylysine.

Pertussis toxin treatment of cells was carried out for 24 h using 0.2 μg ml^{-1} in DMEM containing 0.1% FCS prior to the Ca^{2+} measurement (Freund *et al*., 1994).

Membrane preparation

Crude membranes for the measurement of adenylyl cyclase activity and for binding assays were prepared from fresh or frozen cells, respectively, as described recently (Klotz *et al*., 1998). In brief, cells were homogenized in ice-cold hypotonic buffer (5 mM Tris/HCl, 2 mM EDTA, pH 7.4) and the homogenate was spun for 10 min (4°C) at 1000 × *g*. The crude membrane fraction was sedimented from the supernatant for 30 min at 100,000 × *g*. For binding experiments, the membranes were resuspended in 50 mM Tris/HCl, 10 mM $MgCl_2$, pH 7.4, frozen in liquid nitrogen at a protein concentration of 1–3 mg ml^{-1} and stored at −80°C. For the measurement of adenylyl cyclase activity, crude membranes were prepared with only one centrifugation step. The homogenate from fresh cells was sedimented for 30 min at 54,000 × *g* and the resulting pellet was resuspended in 50 mM Tris/HCl pH 7.4 for immediate use (Klotz *et al*., 1998).

Adenylyl cyclase activity

The procedure followed the protocol described previously (Klotz *et al*., 1998; 1999). Membranes were incubated for 20 min at 37°C in an incubation mixture containing about 150,000 c.p.m. of [α-^{32}P]ATP. The EC_{50}-values for the stimulation of adenylyl cyclase were calculated with the Hill equation. Hill coefficients in all experiments were near unity.

Measurement of intracellular Ca^{2+} and inositol phosphate levels

Concentrations of free intracellular Ca^{2+} was measured in cells grown to confluency on glass coverslips (10 mm diameter) as described by Abd Alla *et al*. (1996) with minor modifications. In brief, the cells were washed twice with HBS buffer (composition in mM: NaCl, 150; KCl, 2.5; $CaCl_2$, 4; $MgCl_2$, 2; glucose, 10; HEPES, 10; pH 7.4), glucose was added before use. For fura-2 loading, cells were incubated in HBS buffer containing 2 μM fura-2/AM and 0.04% (w v^{-1}) of the nonionic detergent pluronic F-127 for 45 min at 37°C. They were washed twice with the buffer and stored in the same buffer at room temperature for another 30 min to allow for complete de-esterification of fura-2/AM. For experiments in the absence of extracelluar Ca^{2+} cells on coverslips were transferred into HBS buffer containing 0.5 mM EGTA instead of $CaCl_2$. Fluorescence was measured with a Perkin-Elmer LS-50 B spectrofluorometer. The excitation wavelength alternated in intervals of 600 ms between 340 and 380 nm. The slit width was 10 nm, and the emission was measured at 510 nm. The intracellular free calcium concentration is given as the ratio of 340/380 nm. Data for each experiment are normalized to the response observed with 10 μM NECA and are reported as percent of response. The data shown were reproduced in at least three independent experiments performed in duplicates.

Inositol phosphate levels were determined according to the procedure as described by Quitterer *et al*. (1999) with minor modifications. Cells were loaded with *myo*-[2-^{3}H]inositol (37 kBq ml^{-1}) for 16 h in inositol-free DMEM supplemented with 0.1% FCS. Before agonist stimulation cells were washed twice with incubation buffer (15 mM HEPES, 137 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.2) and incubated with 10 mM LiCl. Then cells were incubated with NECA for 20 min at 37°C followed by extraction of inositol phsophates as described (Quitterer *et al*., 1999).

[^{3}H]ZM 241385 binding

The binding of [^{3}H]ZM 241385 was measured in membranes prepared from MDA-MB-231 cells as described above. The incubation mixture contained 80 μg of membrane protein in 50 mM Tris/HCl buffer, 10 mM $MgCl_2$, 0.1% BSA, pH 7.4, 0.2 U ml^{-1} adenosine deaminase and the indicated concentrations of radioligand, and was incubated at room temperature.

After 3 h, the samples were filtered over GF/B glassfiber filters and filter-bound radioactivity was determined by liquid scintillation counting. The binding data were analyzed by nonlinear curve fitting with the program SCTFIT (De Lean *et al*., 1982).

Materials

The breast cancer cell lines MCF-7 and MDA-MB-231 were provided by the Institut für Zellbiologie ((Tumorforschung), Universitätsklinik Essen, Germany). PENECA and PHPNECA were synthesised by G. Cristalli (Camerino, Italy) and pharmacologically characterized as described recently (Klotz *et al*., 1999). The nonselective adenosine receptor antagonist 9-ethyl-8-furyladenine (ANR 152) was also synthesised by G. Cristalli (Camerino, Italy). The synthesis and pharmacological characterization will be described elsewhere. Cl-IB-MECA was provided by RBI as part of the NIMH Chemical Synthesis Program. SCH 58261 was kindly provided by P.G. Baraldi (University of Ferrara, Italy). [^{3}H]ZM 241385 and forskolin were from Tocris/Biotrend (Köln, Germany). All other adenosine receptor agonists and antagonists were from Sigma/RBI (Taufkirchen, Germany). Pertussis toxin and thrombin were from Sigma. Fura-2/AM, pluronic F-127, U-73122 and U-73343 were purchased from Calbiochem (Bad Soden, Germany), [α-^{32}P]ATP was from Perkin-Elmer LifeScience (Rodgau, Germany) and 8-Br-cAMP was from Biolog (Bremen, Germany). Cell culture media and FCS were purchased from PanSystems (Aidenbach, Germany). Penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), L-glutamine and G-418 were from Gibco-Life Technologies (Eggenstein, Germany). All other materials were from sources as described earlier (Klotz *et al*., 1998; 1999).

Results

Two human breast cancer cell lines were screened for functional responses to nonselective adenosine receptor agonists. In MDA-MB-231 cells, both a Ca^{2+} signal as well as an increase in cAMP was observed in response to 10 μM NECA or PHPNECA. In contrast, no Ca^{2+} or cAMP signal was detectable in MCF-7 cells (data not shown). Therefore, the following data describe the characterization of adenosine-mediated signals in MDA-MB-231 breast cancer cells only.

Ca^{2+} signaling

Figure 1a shows that 10 μM NECA caused a transient Ca^{2+} signal in MDA-MB-231 cells. A Ca^{2+} signal of almost the same magnitude was observed in the absence of extracellular Ca^{2+} although the signal was fading at a faster rate (Figure 1a). After an initial NECA response, the cells showed a consecutive response of similar magnitude to 10 nM thrombin (Figure 1b). If cells were stimulated by thrombin first, NECA produced a response that was smaller than a first response to NECA and also smaller than the preceding thrombin signal (Figure 1c).

Another nonselective adenosine receptor agonist (PHPNECA, 10 μM) caused a Ca^{2+} signal similar to NECA (Figure 2a). No response was observed for 10 μM of the A_1 selective agonist CCPA (limited selectivity towards A_3), CGS 21680 which mainly acts on A_{2A} and to some extent on A_1 and A_3 receptors (Klotz *et al*., 1998), and the A_3 selective agonists PENECA and Cl-IB-MECA (limited selectivity towards A_1) (Klotz *et al*.,

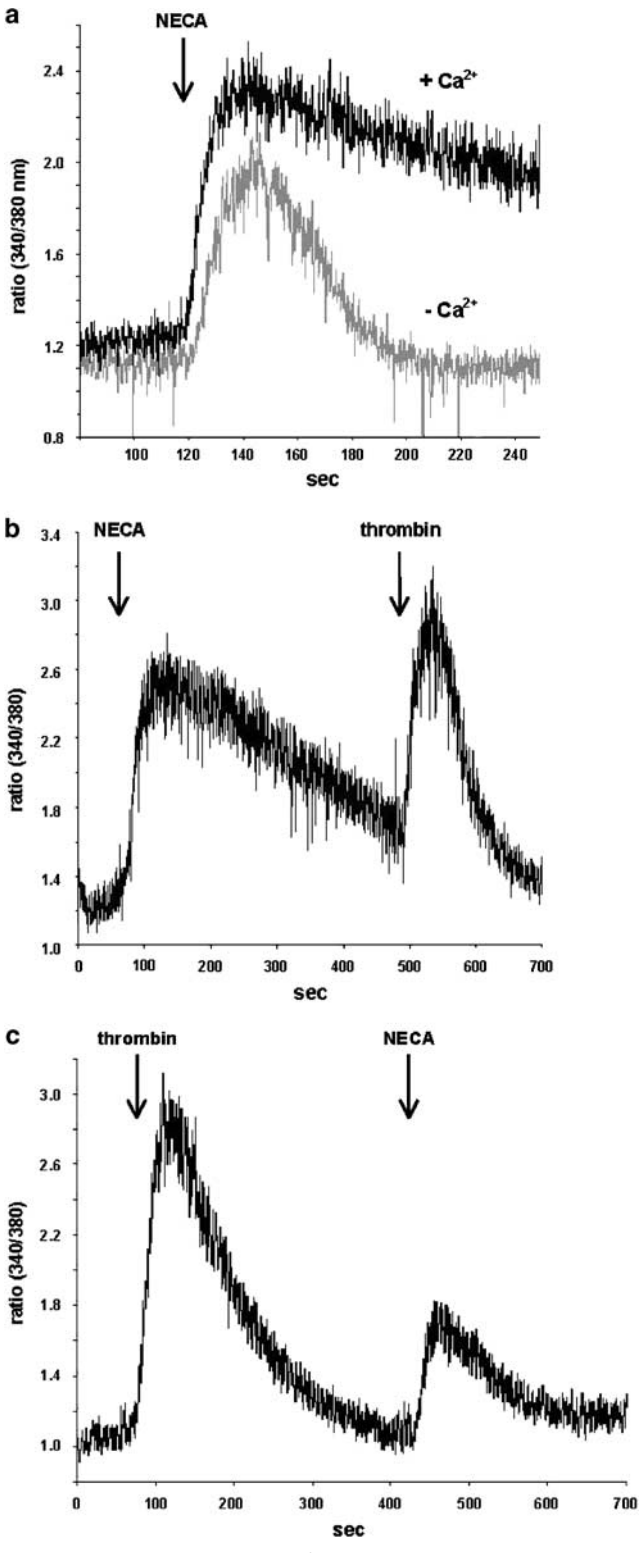

Figure 1 Agonist-mediated Ca^{2+} signal. (a) MDA-MB-231 cells were stimulated with 10 μM NECA in the presence and absence of extracellular Ca^{2+}. (b) NECA stimulation was followed by 10 nM thrombin. (c) Cells were stimulated with thrombin first followed by NECA. For details see text.

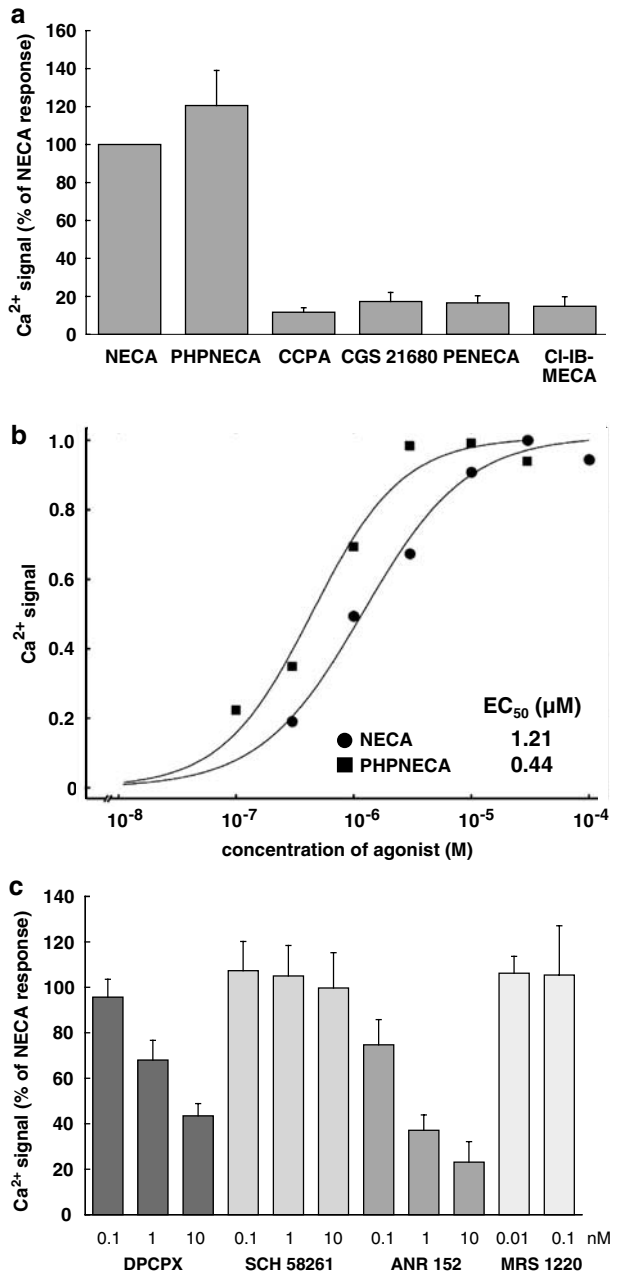

Figure 2 Pharmacological characteristics of the Ca^{2+} signal. (a) The nonselective adenosine receptor agonists NECA and PHPNECA caused a similar Ca^{2+} signal, whereas no response was observed for the subtype selective agonists CCPA, CGS 21680, PENECA and Cl-IB-MECA. The agonist concentration was 10 μM for all compounds. The columns represent means with standard deviation from three experiments performed in duplicate. (b) Concentration–response curves for NECA and PHPNECA. The EC_{50} values for the experiments shown are 1.2 and 0.44 μM, respectively. (c) The A_1/A_{2B} antagonist DPCPX and the nonselective compound ANR 152 antagonized the NECA-induced Ca^{2+} response in a concentration-dependent manner, whereas the A_{2A} selective compound SCH 58261 and the A_3 selective compound MRS 1220 had no effect. Each column represents the means of two experiments with standard deviations.

1999). Figure 2b shows the concentration–response curves for NECA and PHPNECA. The EC_{50} values for these two nonselective agonists were 1250 and 530 nM, respectively (Table 1).

Table 1 Functional responses to adenosine receptor agonists in MDA-MB-231 cells

	Adenylyl cyclase	*Ca^{2+} signal*	*PI-response*
NECA	1130 (1080–1200)	1250 (890–1760)	5400 (3020–9700)
PHPNECA	340 (280–400)	530 (320–890)	n.d.

EC_{50}-values (nM, with 95% confidence limits in parentheses; $n=3$) are shown for A_{2B} receptor-mediated stimulation of adenylyl cyclase, Ca^{2+} signal and PI-response and were determined as described in Methods.
n.d. = not determined.

The NECA response was antagonized in a concentration-dependent manner by the A_1/A_{2B} antagonist DPCPX and the nonselective antagonist ANR 152. The subtype selective antagonists SCH 58261 (A_{2A}) and MRS 1220 (A_3) had no effect on the NECA-induced Ca^{2+} signal (Figure 2c).

Activation of adenylyl cyclase

The pharmacological profile of the NECA-induced Ca^{2+} signal suggested that the response was mediated *via* the A_{2B} adenosine receptor that normally mediates a stimulation of adenylyl cyclase. Therefore, we tested the effect of the nonselective adenosine receptor agonists NECA and PHPNECA on adenylyl cyclase activity. Both agonists caused a similar increase of cAMP production (Figure 3a). CCPA, CGS 21680, PENECA and Cl-IB-MECA did not cause a cAMP response. As stimulation of A_1 and A_3 receptors would elicit an inhibitory signal, the effect of respective agonists on forskolin-stimulated adenylyl cyclase activity was also tested. In Figure 3a it is shown that CCPA, PENECA and Cl-IB-MECA did not inhibit forskolin-stimulated adenylyl cyclase.

The concentration–response curves in Figure 3b show that NECA and PHPNECA cause an about three-fold increase in adenylyl cyclase activity. The EC_{50} values for the cyclase stimulation is 1130 and 340 nM for NECA and PHPNECA, respectively (Table 1).

Investigation of the signaling cascade leading to a Ca^{2+} signal

In order to identify the signaling cascade leading to the A_{2B} receptor-mediated Ca^{2+} response, possible pathways were probed. First, the effect of NECA on production of inositol phosphates was investigated. As shown in Figure 4, NECA caused a concentration-dependent increase of inositol phosphates suggesting a role of phospholipase C for the observed Ca^{2+} signal. The EC_{50} value for the increase of inositol phosphates was 5400 nM (Table 1).

In Figure 5, it is demonstrated that 10 μM of the phospholipase C inhibitor U-73122 almost completely blocked the NECA-induced Ca^{2+} signal whereas the inactive analog U-73343 showed no effect, confirming a role for phospholipase C. Both the cell-permeable cAMP analog 8-Br-cAMP (1 mM) and the adenylyl cyclase stimulator forskolin (10 μM) had no effect on the NECA signal and forskolin caused only a minor Ca^{2+} signal on its own. Inactivation of G_i with pertussis toxin also did not affect the NECA-induced Ca^{2+} signal in MDA-MB-231 cells. It did, however, inhibit the thrombin signal by about 50% (Figure 5).

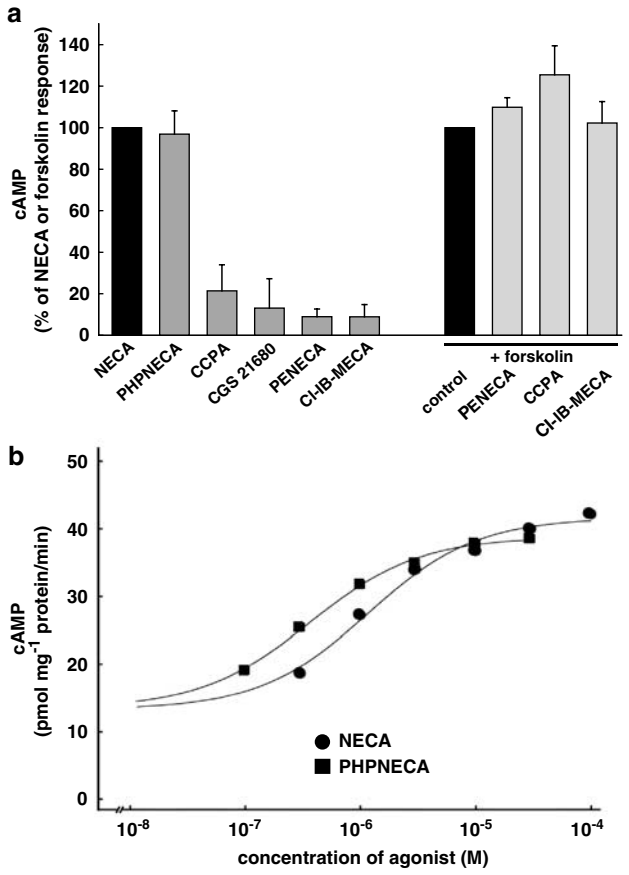

Figure 3 Pharmacological characteristics of the agonist-mediated adenylyl cyclase activation. (a) The nonselective adenosine receptor agonists NECA and PHPNECA caused a similar increase of the activity of adenylyl cyclase, whereas no response was observed for the subtype selective agonists CCPA, CGS 21680, PENECA and Cl-IB-MECA. CCPA, PENECA and Cl-IB-MECA as agonists at the inhibitory receptor subtypes A_1 and A_3, respectively, did not inhibit adenylyl cyclase activity after stimulation by 10 μM forskolin. The agonist concentration was 10 μM for all compounds. Each column represents the mean of three to four experiments with standard deviations. (b) Concentration–response curves for NECA and PHPNECA. The EC_{50} values for the experiments shown are 1.2 and 0.36 μM, respectively.

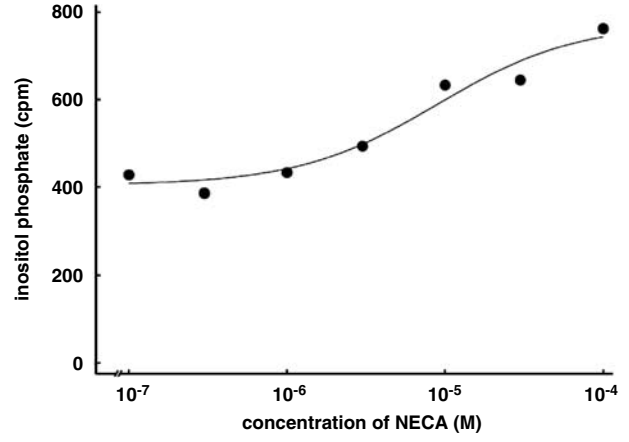

Figure 4 NECA-stimulated accumulation of inositol phosphates. The nonselective adenosine receptor agonist NECA caused a concentration-dependent increase of inositol phosphates. The EC_{50} value in the single experiment shown here was 5.5 μM.

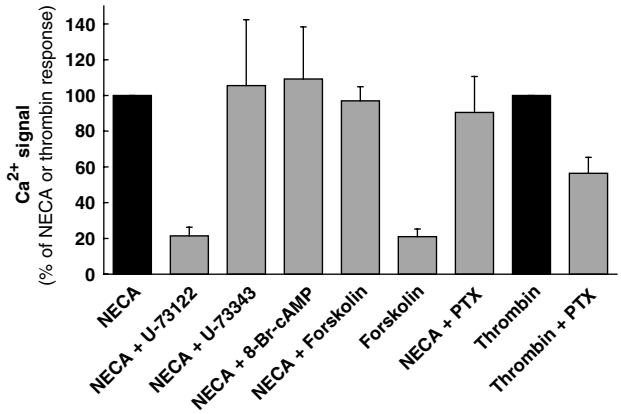

Figure 5 Investigation of the signaling cascade leading to intracellular Ca^{2+} accumulation. The PLC inhibitor U-73122 (10 μM) blocked the NECA-stimulated Ca^{2+} signal demonstrating that the A_{2B} receptor-mediated effect on intracellular Ca^{2+} requires PLC. The inactive analog U-73343 (10 μM) showed no effect. Forskolin (10 μM) or 8-Br-cAMP (1 mM) did not modify the NECA effect on Ca^{2+} levels, forskolin caused only a minor effect on intracellular Ca^{2+} on its own. Pertussis toxin (PTX, 0.2 μg ml^{-1} for 24 h) had no effect on the NECA-induced Ca^{2+} signal while a 50% inhibition of the signal was caused by 10 nM thrombin. The columns represent means with standard deviations of three to four experiments. For further details see text.

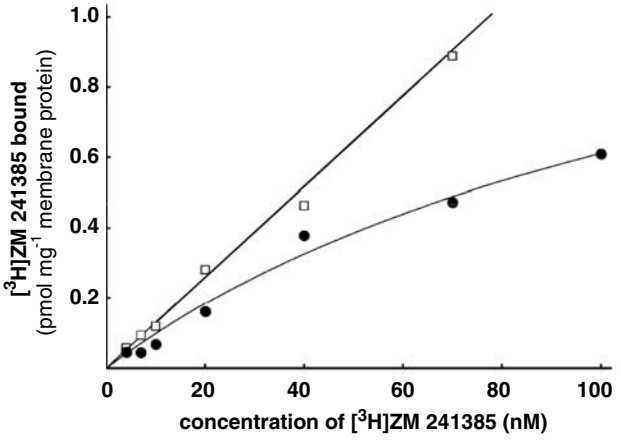

Figure 6 Saturation binding with [^{3}H]ZM 241385. The graph shows the data from a single experiment with each point measured in triplicate. The K_D and B_{max} values are 140 nM and 1480 fmol mg^{-1} membrane protein, respectively. See Table 2 for data in detail.

Radioligand binding with [^{3}H]ZM 241385

Although only poor radioligands for A_{2B} adenosine receptors are available with K_D values that allow to detect receptors only if expressed at very high level, for example, in stably transfected HEK 293 cells (Ji & Jacobson, 1999; Linden *et al*., 1999), binding experiments were attempted. As shown in Figure 6, saturable binding of [^{3}H]ZM 241385 was detected suggesting high A_{2B} receptor expression in MDA-MB-231 cells. A K_D value of 87 nM and a B_{max} value of 1600 fmol mg^{-1} membrane protein was determined in this cell line (Table 2). The nonspecific binding was fairly high and amounted to about 50–75% at K_D value. Specific binding was inhibited by 10 μM of the nonselective antagonist ANR 152 but not by 10 μM of the A_{2A} agonist CGS 21680 (data not shown).

Table 2 Saturation experiments with [^{3}H]ZM 241385

	K_D (nM)[a]	B_{max} (fmol mg^{-1} protein)[b]
[^{3}H]ZM 241385	87.0 (50.3–150)	1600 (±243)

[a]95% confidence limit is in parenthesis.
[b]s.e.m. value is in parenthesis.

Discussion

A_3 adenosine receptors have been suggested to play an important role in control of growth and proliferation of tumor cells (Madi *et al*., 2003; 2004; Fishman *et al*., 2004). Some studies utilizing IB-MECA provide indirect evidence for the participation of an A_3 receptor in the regulation of cell growth and proliferation in human breast cancer cells (Lu *et al*., 2003; Panjehpour & Karami-Tehrani, 2004). In this study we investigated, therefore, the presence of functionally significant adenosine receptor subtypes in two human breast cancer cell lines. As parameters for functional adenosine-mediated responses, the effects of nonselective agonists on intracellular Ca^{2+} levels and on the activity of adenylyl cyclase were determined. The estrogen receptor-positive MCF-7 cells did not show any functional response to nonselective adenosine receptor agonists like NECA. On the other hand, the estrogen receptor-negative cell line MDA-MB-231 responded to NECA with an increase in cAMP and a pronounced Ca^{2+} signal.

The pharmacological characteristics of both the cAMP response and the Ca^{2+} signal were indicative of the sole presence of an A_{2B} adenosine receptor in MDA-MB-231 cells. This was evidenced by the lack of a stimulatory effect on cAMP levels by the A_{2A} agonist CGS 21680 as well as a lack of an inhibition by A_1 or A_3 selective agonists like CCPA or PENECA of forskolin-stimulated cyclase activity. An identical activation pattern was observed for the Ca^{2+} signal indicating that both signals were mediated by the same receptor subtype. The nonselective compounds NECA and PHPNECA were the only agonists that caused a functional response suggesting that they signal *via* the A_{2B} adenosine receptor. The antagonist profile for the inhibition of the Ca^{2+} signal clearly confirmed the notion that it was mediated *via* an A_{2B} subtype.

Although A_{2B} adenosine receptors were pharmacologically distinguished from the A_{2A} subtype over two decades ago (Bruns, 1980) and the human subtype was cloned in 1992 (Pierce *et al*., 1992), their function is still elusive. One of the problems is that selective ligands are still scarce, and, in particular, no A_{2B}-selective agonists with high potency are known so far. It may well be that high-affinity ligands are hard to find because adenosine exhibits only low affinity for the A_{2B} receptor (Fredholm *et al*., 2001b). Therefore, physiological levels of adenosine are not sufficient to stimulate this subtype. Even the most potent agonists like PHPNECA exhibit EC_{50}-values at the A_{2B} receptor around 1 μM only, but are more potent at other adenosine receptor subtypes (Klotz *et al*., 1999). Some A_{2B}-selective antagonists have been reported (Kim *et al*., 1999; Hayallah *et al*., 2002; Baraldi *et al*., 2004); however, they did not reveal more insight into the A_{2B} receptor function as physiological adenosine levels are much too low for a tonic effect to occur that could be blocked by selective antagonists. Consistent with an EC_{50}-value of 420 nM of PHPNECA for the stimulation of adenylyl cyclase activity in CHO cells transfected with the human A_{2B} receptor (Klotz *et al*., 1999), similar potencies were found for the stimulation of adenylyl cyclase activity (EC_{50} 340 nM) and a Ca^{2+} signal (EC_{50} 530 nM) in MDA-MB-231 cells. Similarly, the NECA potency for cyclase activation (EC_{50} 1130 nM) was in close agreement with previous data with transfected cells from our (EC_{50} 2360 nM; Klotz *et al*., 1998) and other laboratories (EC_{50} 1230 nM; Alexander *et al*., 1996).

Further evidence for the existence of an A_{2B} adenosine receptor in MDA-MB-231 cells came from binding studies. We could not detect any specific binding of [^{3}H]CCPA, [^{3}H]NECA or [^{3}H]CGS 21680 (data not shown), but we were able to show specific and saturable binding of [^{3}H]ZM 241385. Although originally introduced as an A_{2A} selective radioligand, it turned out to have significant affinity for the A_{2B} subtype (Ji & Jacobson, 1999). From saturation experiments, we determined a K_D-value of 87 nM, which is in agreement with a reported A_{2B} K_D-values of 34 nM for this ligand (Ji & Jacobson, 1999). With 1600 fmol mg^{-1} membrane protein, we found a receptor density for an endogenous A_{2B} adenosine receptor of about one-third of the B_{max} value reported for an overexpressing HEK cell line (Ji & Jacobson, 1999). MDA-MB-231 cells seem to be the first cell line expressing an endogenous A_{2B} receptor at levels high enough to be detected in radioligand binding.

Both A_{2A} and A_{2B} receptors couple to G_s and cause a stimulation of adenylyl cyclase. Although unusual for a stimulatory receptor, reports in the literature suggest that the A_{2B} subtype may also mediate a Ca^{2+} signal. In CHO cells transfected with the human A_{2B} receptor, we could not detect such a response although the same cells transfected with A_3 receptors mediated a Ca^{2+} response *via* $G_{i/o}$ (Englert *et al*., 2002). For the A_{2B}-mediated Ca^{2+} responses so far described in the literature, different signaling pathways were suggested. The first report by Feoktistiov *et al*. (1994) suggests that A_{2B} receptors in human erythroleukemia cells are capable of potentiating a Ca^{2+} signal elicited by other ligands like thrombin or PGE_1 involving G_s. On the other hand, the same group identified an A_{2B} receptor in human mast cells, which was shown to cause a rise of intracellular Ca^{2+} levels and inositol phosphates in a pertussis toxin-insensitive manner, suggesting a G_q-mediated pathway (Feoktistov & Biaggioni, 1995). In Jurkat cells, an A_{2B} receptor-mediated increase in intracellular Ca^{2+} was demonstrated by Mirabet *et al*. (1997) independent of inositol phosphates. Auchampach *et al*. (1997) and Gao *et al*. (1999) found an A_{2B}-mediated increase in intracellular Ca^{2+} in canine mastocytoma cells and HEK-293 cells, respectively, presumably *via* a G_q-coupled pathway. The A_{2B}-mediated Ca^{2+} mobilization in human breast cancer cells MDA-MB-231 in our study was clearly not mediated by an increase in cAMP because forskolin did not mimic the NECA effect on intracellular Ca^{2+}. It was also independent of a pathway *via* $G_{i/o}$ and $\beta\gamma$ as pertussis toxin did not inhibit the Ca^{2+} signal. However, inhibition of phospholipase C with the specific inhibitor U-73122 abolished the NECA effect on intracellular Ca^{2+} levels, suggesting that the signaling pathway in the MDA-MB-231 cells involved $G_{q/11}$ (Figure 7).

From Figure 1a, it is obvious that activation of the A_{2B} receptor results in both Ca^{2+} release from intracellular stores as well as Ca^{2+} influx through plasma membrane channels. Such entry of external Ca^{2+} is observed in response to receptor-mediated emptying of intracellular stores in many

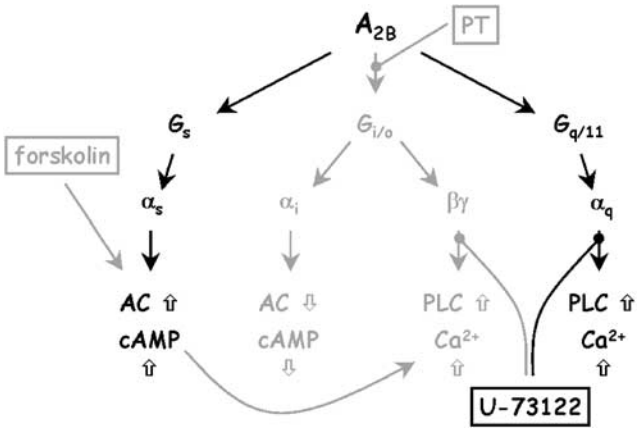

Figure 7 Possible pathways leading to effector activation by A_{2B} adenosine receptors. In MDA-MB-231, both activation of adenylyl cyclase as well as a PLC-mediated increase of intracellular Ca^{2+} was shown. The Ca^{2+} signal proceeds most likely *via* a $G_{q/11}$-mediated pathway. Pathways excluded in this study are shown in gray. For further details, see text.

systems and is designated as store-operated Ca^{2+} entry. It appears that an extended family with various types of store-operated Ca^{2+} channels is responsible for this type of Ca^{2+} influx (for review, see Clapham *et al*., 2003).

A_{2B} adenosine receptors have been identified in several cancerous cell lines (Feoktistov *et al*., 1994; Feoktistov & Biaggioni, 1995; Mirabet *et al*., 1997; Zeng *et al*., 2003). It is striking that in all these cases, an A_{2B} receptor-mediated Ca^{2+} signal has been found. Ca^{2+} is an intracellular signal that is involved in numerous cellular processes including cell differentiation and proliferation, activation of transcription factors, apoptosis and control of malignancy (for review, see Berridge *et al*., 2000). It is, therefore, conceivable that A_{2B} adenosine receptors do play a role in the control of tumor growth, and development as adenosine levels in solid tumors may reach micromolar levels (Blay *et al*., 1997) and are thus high enough to stimulate the low-affinity A_{2B} subtype. Both activation of PLC and increasing intracellular Ca^{2+} levels have been thought to play a role in cell transformation and growth (Berridge *et al*., 2000) and, consequently, blockade of A_{2B} adenosine receptors may help to control tumor growth. It is interesting to note that the level of the adenosine-producing ecto-5′-nucleotidase was recently shown to be inversely related to estrogen receptor expression (Spychala *et al*., 2004). These authors concluded that ecto-5′-nucleotidase might serve as a marker for more aggressive estrogen receptor-negative breast carcinoma. Such cells expressing high levels of ecto-5′-nucleotidase would produce the highest levels of adenosine and it may be in these cells that blocking A_{2B} adenosine receptors provides a therapeutic option to control tumor progression. The potential role of G_s-mediated cAMP signaling *via* A_{2B} receptors in the context of cell transformation and tumor proliferation remains unclear at present.

In summary, we have functionally characterized an endogenous A_{2B} adenosine receptor in the estrogen receptor-negative human breast cancer cell line MDA-MB-231. These cells may serve as a model for native human A_{2B} subtype as they express high receptor levels with functional effector coupling to two different signaling pathways. The A_{2B} receptors mediate a stimulation of adenylyl cyclase, but are in addition coupled to a Ca^{2+} signal. The Ca^{2+} response requires activation of phospholipase C and appears to be mediated *via* $G_{q/11}$ pathway. Further work will be required to understand the role of the A_{2B} adenosine receptors in tumor growth and development.

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