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Calcium mobilization in Jurkat cells via A_{2b} adenosine receptors

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1 A functional study of cell surface A_{2b} adenosine receptors was performed on the T cell leukaemia line, Jurkat.

2 A_{2b} receptors were coupled both to the adenylate cyclase system and to intracellular calcium channels. In fact, the agonist of A_{2b} receptors, 5′-*N*-ethylcarboxamidoadenosine (NECA), led to a transient accumulation of intracellular calcium by an inositol phosphate-independent mechanism.

3 The NECA-induced accumulation of cGMP was not responsible for the calcium mobilization via A_{2b} receptors.

4 The calcium response elicited by activation of A_{2b} receptors was independent of that evoked by activation of the T cell receptor.

5 These findings not only delineate a novel transduction mechanism for adenosine but also support a specific role for adenosine in modulating signals elicited via the T cell receptor.

Keywords: A_{2b} adenosine receptors; calcium; calcium channels; cGMP; Jurkat cells; T lymphocytes; T cell receptor

Introduction

Adenosine is an important modulator of the function of many cell types. This modulation is mediated by G protein-coupled adenosine receptors, four of which (A_1, A_{2a}, A_{2b} and A_3) have been cloned (Pierce *et al*., 1992; Rivkees & Reppert, 1992; Palmer & Stiles, 1995). Classification of adenosine receptors was first established on the basis of the changes associated with the activity of adenylate cyclase. According to this criterion, the A_1 subtype mediates decreases in cAMP levels by means of a signal transduced by inhibitory, pertussis toxin sensitive, G proteins whereas the A_2 subtypes mediate increases in cAMP by means of cholera toxin sensitive mechanisms (van Calker *et al*., 1979; Londos *et al*., 1980). Moreover, although there is no clear evidence of functional coupling of A_3 receptors to the adenylate cyclase system, activation of recombinant A_3 receptors expressed in CHO cells leads to inhibition of the cAMP response elicited by forskolin (Zhou *et al*., 1992).

In lymphocytes, adenosine analogues produce an accumulation of intracellular cAMP, thus suggesting that the adenosine receptors are of the A_2 subtypes (Nordstedt *et al*., 1987). By means of the polymerase chain reaction, van der Ploegh *et al*. (1996) have identified the presence of mRNA from both the A_{2a} and A_{2b} subtypes in Jurkat T cells. However, the relative proportion of A_{2a} adenosine receptors is low as their activation is insufficient to stimulate adenylate cyclase unless forskolin is also added. Therefore, the main functional A_2 adenosine receptors present in Jurkat cells are of the A_{2b} subclass (Van der Ploegh *et al*., 1996).

Adenosine analogues acting upon A_{2b} receptors produce an increase in intracellular cAMP in a variety of cell systems (Feoktistov & Biaggioni, 1993; Peakman & Hill, 1994; Strohmeier *et al*., 1995). In addition to coupling with adenylate cyclase, A_{2b} receptors can couple with a variety of other signal transduction components. For example, there is a positive modulation of intracellular Ca^{2+} levels mediated by A_{2b} receptors in human erythroleukaemia cells. This modulation is independent of cAMP production but operates through a cholera toxin sensitive mechanism. The increase in intracellular calcium levels is not related to phosphoinositide hydrolysis but to an increase in calcium influx, which is lost in the absence of extracellular calcium (Feoktistov *et al*., 1994). Functional expression of A_{2b} receptors in *Xenopus laevis* oocytes induces calcium-dependent Cl^- conductance that might be related to phospholipase C activation (Yakel *et al*., 1993).

Ca^{2+} is an important second messenger produced in the course of T cell activation. Regulation of intracellular calcium concentrations in lymphoid cells is rather complex. In fact, Guse *et al*. (1993) have demonstrated that four different intracellular Ca^{2+} pools coexist in Jurkat cells. Pool I is agonist- and inositol(1,4,5)P_3-sensitive and contains 23% of the total Ca^{2+} storage capacity. Pool II contains 23% of the intracellular Ca^{2+} and is released by the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin. Pool III contains 23% of the calcium and is caffeine sensitive. In T lymphocytes this reservoir is blocked by ruthenium red and is sensitive to cyclic adenosine diphosphate-ribose, a metabolite of NAD^+ metabolism (Guse *et al*., 1995). A second messenger role of cyclic adenosine diphosphate-ribose requires that its intracellular levels be under the control of an extracellular stimulus. Galione *et al*. (1993) have demonstrated that stimulation of the synthesis of this compound can be achieved by cGMP, which itself is produced in response to a variety of hormones acting at the plasma membrane level. However, it is not known how widespread is this mechanism, which has been demonstrated in sea urchin eggs (Galione *et al*., 1993) and in a neurosecretory cell line (Clementi *et al*., 1996). Finally, pool IV can replenish pool III and is released by the Ca^{2+} ionophore inonomycin. The aim of this paper is to assess whether adenosine can lead to calcium mobilization in T cells and, if so, to identify the pool from which the ion is released. The results provide evidence that adenosine, via A_{2b} adenosine receptors induces the release of calcium by a cGMP- and inositol phosphate-independent mechanism.

Methods

Materials

5′-*N*-ethylcarboxamidoadenosine (NECA), N^6-(R)-phenylisopropyladenosine (R-PIA), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), forskolin, Indo-1/AM, cholera toxin, Triton X-100 and EGTA were purchased from Sigma Co. (St Louis, MO, U.S.A.). 8[4-(2-aminoethyl)amino]carbonyl]methyl]oxy]-phenyl-1,3-dipropylxanthine, commonly known as xanthine amino congener (XAC), N^6-(3-iodobenzyl)-adenosine-5′-*N*-

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methyluronamide (IB-MECA) and 2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-ethylcarboxamidoadenosine (CGS21680) were from Research Biochemicals International (Natick, MA, U.S.A.) and HEPES was from Boehringer Mannheim (Barcelona, Spain). Fura-2/AM, fluo-3/AM and Fura Red/AM were obtained from Molecular Probes (Eugene, OR, U.S.A.) and LY 83583 was from Calbiochem (La Jolla, CA, U.S.A.). [^{3}H]NECA, [^{3}H]R-PIA, [^{3}H]DPCPX and [^{3}H]CGS21680 were from New England Nuclear Research Products (Boston, MA, U.S.A.) and activated pertussis toxin was from ICN Biomedical Inc. (Itisa, Madrid, Spain). 3-(4-hydroxy-5-hydroxymethyl-tetrahydro-furan-2-yl)-3, 6, 7, 8-tetrahydro-imidazo[4,5-d][1,3]diazepin-8-ol (deoxycoformicin), used as potent and irreversible inhibitor of adenosine deaminase, an enzyme which degrades adenosine, was kindly provided by Dr Carol L. Germain (Parke-Davis/Warner Lambert, Ann Arbor, MI, U.S.A.). 5-Amino-9-chloro-2-(2-furanyl)1,2,4-triazolo(1,5-C)quinazolinemonomethanesulphonate (CGS15943), dipyridamole and OKT3 antibody were kindly provided by Dr Richard A. Lovell (Ciba-Geigy Corp., Summit NJ, U.S.A.), Dr Cembrano (Boehringer Ingelheim; Barcelona, Spain) and Dr Terhorst (Beth Israel Hospital, Boston, MA, U.S.A.), respectively. All other products were of the best grade available and were purchased from Merck (Darmstad, Germany) and Sigma. Deionised water further purified with a Millipore Milli-Q system was used throughout.

Cell culture conditions

Jurkat cells were maintained in RPMI 1640 medium (GIBCO; Grand Island, NY, U.S.A.) supplemented with 10% (v:v) foetal calf serum (Boehringer Mannheim), 2 mM L-glutamine and antibiotics (GIBCO; 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} fungizone) at 37°C in a humid atmosphere of 5% CO_2.

Membrane preparations

Cells were harvested and homogenized in ice-cold 50 mM Tris-HCl buffer pH 7.4 with a Polytron (Kinematica). After 30 min on ice, the homogenate was centrifuged at 900 *g* for 10 min to remove nuclei and the supernatant was then centrifuged at 100 000 *g* for 45 min (4°C). The resulting membrane pellet was resuspended in 50 mM Tris-HCl buffer and recentrifuged under the same conditions. The pellet was washed once more and stored at −70°C until use, or resuspended in the same buffer solution for immediate use.

Protein determination

Protein was determined by the bicinchoninic acid method (Pierce) as described by Sorensen & Brodbeck (1986).

Binding experiments

Membrane pellets were resuspended at 0.3 mg protein/ml in 50 mM Tris-HCl buffer pH 7.4 containing 0.2 U ml^{-1} adenosine deaminase (Boehringer Mannheim). After 30 min at room temperature, ligand binding was measured by incubating the membrane suspension with the radiolabelled compound in the presence or absence of different competitors for 2 h at room temperature. Free and bound ligands were separated by rapid passage of 500 μl aliquots through GF/C filters (Whatman, Kent, U.K.), which were subsequently washed in 10 ml of ice-cold Tris-HCl buffer. Filters were shaken overnight in 10 ml of Formula 989 scintillation cocktail (New England Nuclear) and radioactivity was counted using a Packard 1600 TRI-CARB scintillation counter with a 50% efficiency. Non-specific binding in the case of [^{3}H]NECA was determined in the presence of 250–500 μM unlabelled NECA. For other radioligands, non-specific binding was quantified in the presence of 200- to 300-fold excess of the unlabelled reagent.

Analysis of intracellular Ca^{2+} by double wavelength fluorimetry

Jurkat cells 5×10^6/ml were loaded with 5 μM Indo-1/AM in HBSS buffer (140 mM NaCl; 5 mM KCl, 1 mM $MgCl_2$, 1 mM $MgSO_4$, 1.2 mM $CaCl_2$, 10 mM HEPES, 5 mM glucose, 0.3 mM KH_2PO_4 and 2 mM Na_2HPO_4) pH 7.0 for 30 min at 37°C. Then an equal volume of HBSS pH 7.4, containing 10% heat inactivated fetal calf serum, was added and the cell suspension was incubated for an additional 30 min. After washing with HBSS pH 7.2 containing 5% heat inactivated foetal calf serum and 10 μg ml^{-1} bovine pancreas deoxyribonuclease I (Sigma), cells were resuspended at 5×10^6 cells/ml and maintained at room temperature in the dark until used for $[Ca^{2+}]_i$ determinations. Just before the analysis, cells were diluted to 1×10^6 cells/ml and warmed to 37°C. Fluorescence was monitored with a RF-5000 Shimadzu spectrofluorimeter in cuvettes thermostatically controlled at 37°C and continuously stirred. The cell suspension was excited at 355 nm and fluorescence emissions were detected at 405 (Ca^{2+}-bound dye) and 485 nm (Ca^{2+}-free dye). Final $[Ca^{2+}]_i$ values were calculated from the ratio of emission fluorescences (405/485 nm) using the equation described by Grynkiewick *et al*. (1985), with a K_d value of 250 nM for Indo-1. The R_{max} value was obtained by lysing the cells with 0.1% Triton X-100, followed by an addition of excess EGTA for R_{min}. For determinations without extracellular Ca^{2+}, no heat inactivated foetal calf serum was present in the final suspension buffer and 4 mM EGTA was added to the cuvettes. R_{max} was obtained after addition of 0.1% Triton X-100 and 10 mM $CaCl_2$ and R_{min} by adding an excess of EGTA. Fura-2 was used to overcome interference in Indo-1 fluorescence detection caused by the addition of LY 83583 to cuvettes. In these experiments, 5×10^6 cells/ml were loaded with 5 μM Fura-2/AM in RPMI 1640 for 30 min at 37°C, washed twice in HBSS buffer (120 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 0.4 mM $CaCl_2$, 10 mM HEPES, 10 mM $NaHCO_3$, 10 mM glucose and 5 mM Na_2HPO_4) pH 7.4, and incubated in buffer for an additional 30 min. Finally, cells were washed once, resuspended at 5×10^6 cells/ml and used as described for Indo 1-loaded cells with excitation wavelengths of 340 and 380 nm and emission wavelength of 505 nm.

Analysis of intracellular Ca^{2+} and DNA content by flow cytofluorimetry

Jurkat cells were loaded with Indo-1/AM as described above. The cell suspension was excited with the 334–364 nm lines of an argon ion laser and the ratio between the emission fluorescences (395/525 nm) was detected at different times with an Epics Elite flow cytometer (Coulter Corporation; Hialeah, FL, U.S.A.). The percentage of cells responding to the stimulus was measured at the time when the calcium mobilization was maximum. In time-course experiments flow cytometric data from individual cells analysed at short time intervals were grouped. The number of cells responsive or unresponsive to the stimulus was performed according to the fluorescence ratio at the time when the maximum calcium mobilization is achieved. Before the stimulus cells display a low fluorescence ratio; therefore, cells whose fluorescence ratio do not significantly change after the stimulus are considered unresponsive whereas the remaining are considered responsive. For simultaneous analysis of DNA content and intracellular Ca^{2+} levels, 1×10^7 cells/ml were loaded with 4 μM fluo-3/AM and 10 μM Fura Red/AM in RPMI 1640 for 30 min at room temperature. After washing twice in RPMI 1640, cells were resuspended at 5×10^6 cells/ml; 30 min before the analysis, cells were diluted to 1×10^6 cells/ml with RPMI 1640 and incubated with 4 μM Hoechst 33342 (Sigma) at 37°C. Fluo-3 and Fura Red were excited at 488 nm, and the ratio between their emission fluorescences (525 nm for Fluo 3 and 675 nm for Fura Red) was collected. Hoechst 33342 was excited at 334-364 nm and fluorescence was detected at 395 nm. To avoid fluorescence interference between Hoechst 33342 and Fluo 3, both lasers

(488 and 334–364 nm) excited every cell at a different time. Signals obtained from the two impacts were simultaneously processed by the 'gate amplifier' option of the flow cytometer. This allows for the analysis of two different signals obtained from the same cell at different times.

Inositol phosphate determination

Cells (2.5×10^5 cell/ml) were loaded with 2 μCi/ml [^{3}H]myoinositol (102 Ci/mmol; Amersham Iberica, Madrid, Spain) for 48 h at 37°C in inositol-free RPMI 1640 (Sigma), 10% inositol-free foetal calf serum, 2 mM L-glutamine and antibiotics. Cells were washed twice in medium and incubated at 5×10^6 cells/ml in inositol-free RPMI 1640 containing 10 mM LiCl and 10 mM HEPES for 10 min. Cells were then aliquoted into tubes (200 μl/tube) containing 5 μl of the appropriate stimuli and incubated at room temperature for 20 min. The reaction was stopped by adding 75 μl of ice-cold 2 M perchloric acid and then leaving the mixture on ice for 30 min. After neutralization with the appropriate volume of 1 M KOH/1 M Tris/60 mM EDTA, the suspensions were centrifuged at 12 000 *g* for 10 min at 4°C in a microfuge; 10 ml of H_2O was added to 350 μl of supernatants and the diluted samples were applied to 1 ml of Dowex AG1-X8 ion exchange columns (BIO-Rad Laboratories, Hercules, CA, U.S.A.). After rinsing in 20 ml of H_2O, total [^{3}H]inositol phosphates were collected by eluting the columns with 15 ml of 100 mM HCOOH/1M NH_4COOH. Then 3 ml of eluates were added to 7 ml of Optiphase Hisafe 3 scintillation liquid (Pharmacia Wallac, Turku, Finland) and radioactivity was counted.

Determination of cyclic AMP and cyclic GMP levels

For cAMP determination, Jurkat cells were preincubated at 2×10^6 cells/ml in HBSS containing 30 μM RO-20-1724 (Calbiochem) for 10 min at 37°C; 250 μl of the cell suspension were added to tubes containing 2.5 μl of the appropriate stimuli and, after a 10 min incubation at 37°C, 500 μl of ice-cold ethanol were added to stop the reaction. Samples were centrifuged at 2000 *g* for 20 min at 4°C and supernatants were lyophilized. cAMP levels were determined using an enzyme-immunoassay kit from Amersham. cGMP levels were also determined using an enzyme-immunoassay kit (Amersham), following the acetylation protocol. Samples were obtained as described for cAMP quantification, with the exception that 8×10^6 cells/ml were preincubated in HBSS containing 25 μM dipyridamole for 15 min at 37°C before the addition of stimuli.

Results

Characterization of cell surface adenosine receptors in Jurkat cells

Adenosine receptors were characterized in Jurkat cells through experiments measuring displacement of [^{3}H]NECA binding to cell membranes. Jurkat cell membranes were able to specifically bind NECA with a K_d value in the range 1.2–1.5 μM. Specific binding was the same when [^{3}H]NECA binding was displaced by either 250 μM NECA or 1 mM R-PIA thus indicating the absence of any low-affinity NECA binding protein (adenotin, Fein *et al*., 1994) in these membranes. Whereas the specific binding of 49 nM [^{3}H]NECA was 1.1 ± 0.5 pmol/mg membrane protein, the specific binding of either 2.4 nM [^{3}H]DPCPX, 8.5 nM [^{3}H]R-PIA or 12.5 nM [^{3}H]CGS21680 was negligible. The specific binding of 32.5 nM [^{3}H]DPCPX was very low (0.3 ± 0.2 pmol mg^{-1} protein). The rank order of potency of purine compounds as displacers of 50 nM [^{3}H]NECA binding was (Figure 1, $pIC_{50} \pm$ s.d. is given in parentheses): NECA (5.8 ± 0.1) > DPCPX (5.2 ± 0.2) > R-PIA (4.1 ± 0.1) > CGS21680 and IB-MECA (<4). These data and the NECA-induced accumulation of cAMP (from 2.2 ± 0.9 to 8 ± 2 pmol cAMP/10^6 cells at a concentration of 100 μM) indicate the presence of A_{2b} receptors functionally coupled to adenylate cyclase.

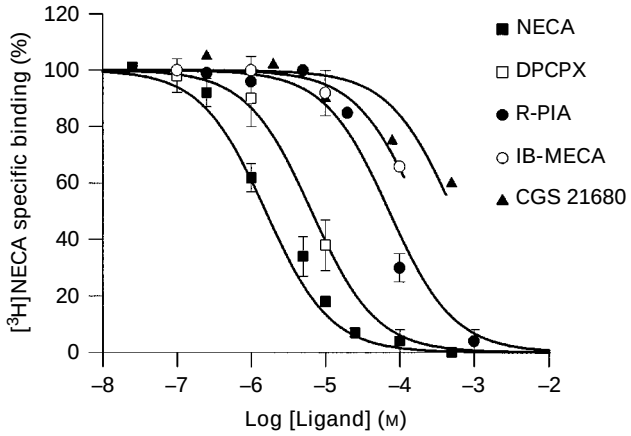

Figure 1 Displacement of [^{3}H]NECA binding by different reagents. Membranes (0.3 mg of protein/ml) were incubated at room temperature with 50 nM [^{3}H]NECA in 50 mM Tris-HCl buffer (pH 7.4) in the absence or presence of increasing amounts of the indicated reagent. After 2 h of incubation, free radioligand was separated from bound radioligand as indicated in Methods. Data are the mean of quadruplicates from a representative experiment. Higher concentrations of some of the compounds could not be achieved due to solubility problems.

Calcium mobilization via cell surface A_{2b} adenosine receptors

Addition of NECA to cells led to a transient accumulation of intracellular Ca^{2+}, both in the presence and in the absence of EGTA, which chelates extracellular calcium (Figure 2). Similar results to those obtained in the presence of EGTA were obtained in a Ca^{2+}-free buffer (data not shown). When the experiment was performed in the presence of EGTA, the accumulation was smaller than that obtained in the absence of EGTA and [Ca^{2+}] levels returned to baseline in approximately 2 min (Figure 2a). Therefore, the NECA-induced release of calcium was from a Ca^{2+}-regulated intracellular pool The $pEC_{50} \pm$ s.d. value of the NECA effect was 5.4 ± 0.1 in the absence of EGTA and 5.55 ± 0.05 in its presence (Figure 2b). These values were similar to the pK_d for [^{3}H]NECA binding to the A_{2b} receptors present in the cell membranes. The order of potency of agonists on the calcium response (Figure 3) was NECA > adenosine > R-PIA > CPA > CGS21680 = IB-MECA. DPCPX, a non-selective antagonist of A_{2b} receptors, blocked the NECA-induced accumulation of calcium. The antagonism was dose-dependent with an pIC_{50} value of 6.4 μM (Figure 4). The order of potency of antagonists upon blocking of the NECA-induced calcium mobilization was: CGS15943 > XAC > DPCPX (Figure 4). The order of potency of both agonists and antagonists is compatible with the presence of an A_{2b} receptor (Peakman & Hill, 1994, Alexander *et al*., 1996). Pretreatment of Jurkat cells with NECA led to desensitization of the adenosine receptors present on the cell surface and therefore the calcium signal originated by activation of these receptors diminished. The opposite occurred upon treatment with DPCPX, which led to an upregulation of receptors and to a marked increase in the intracellular calcium concentration in response to NECA (Figure 5). Taken together these results suggest that the effect was receptor-mediated and that the receptors involved were of the A_{2b} subclass.

The NECA-induced increase in intracellular calcium was monitored by flow cytometry. Cells loaded with INDO-1 were triggered by NECA or by the anti-CD3 antibody OKT3, which initiates T cell activation responses via calcium mobilization and protein tyrosine phosphorylation (Weiss & Littman, 1994; Cantrell, 1996). The fluorescence of individual cells was ana-

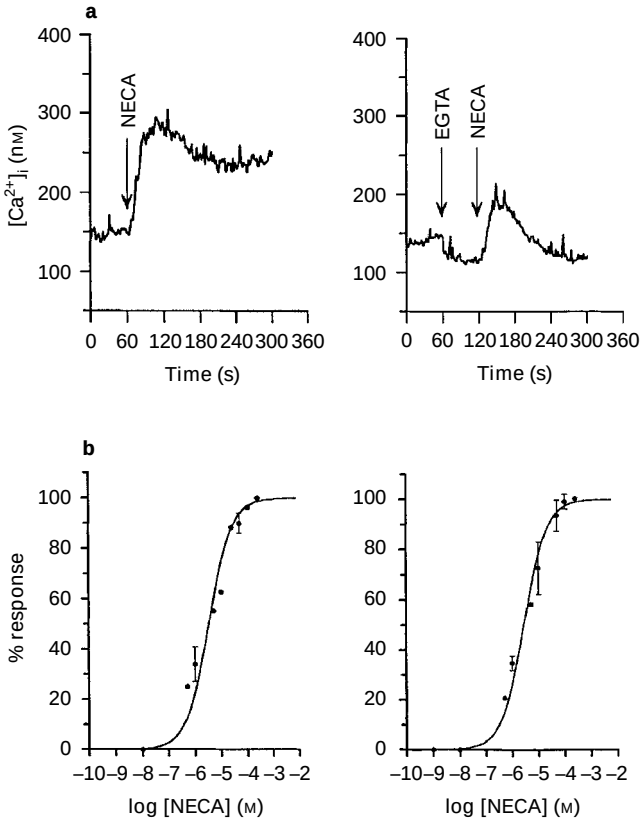

Figure 2 NECA-induced calcium response in Jurkat cells. Cells were loaded with Indo-1 in Ca^{2+}-containing medium and fluorescence was measured as described in Methods. (a) Calcium response in the absence (left) or in the presence (right) of 4 mM EGTA. (b) Dose–response curves for calcium mobilization in the absence (left) or in the presence (right) of 4 mM EGTA. Data are expressed as the percentage of the maximal response achieved in each experiment and are the mean of three experiments ± s.d. The pEC_{50} values were 5.5 ± 0.1 in the absence of EGTA and 5.6 ± 0.1 in the presence of EGTA.

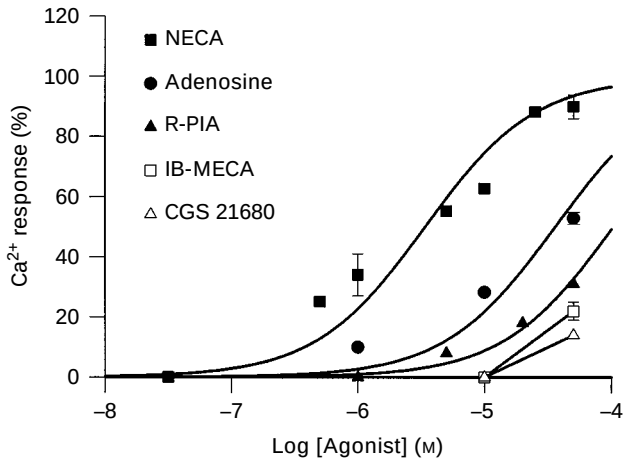

Figure 3 Order of potency of ligand-induced calcium response in Jurkat cells. Cells were loaded with Indo-1 in Ca^{2+}-containing medium and fluorescence was measured in the absence of EGTA as described in Methods. Maximum responses for each ligand at each concentration were compared with those found with a maximal dose of NECA. Data (mean of three experiments ± s.d.) are given in percentages. Adenosine-induced calcium response was assayed in the presence of 1 μM deoxycoformycin to inhibit adenosine deaminase.

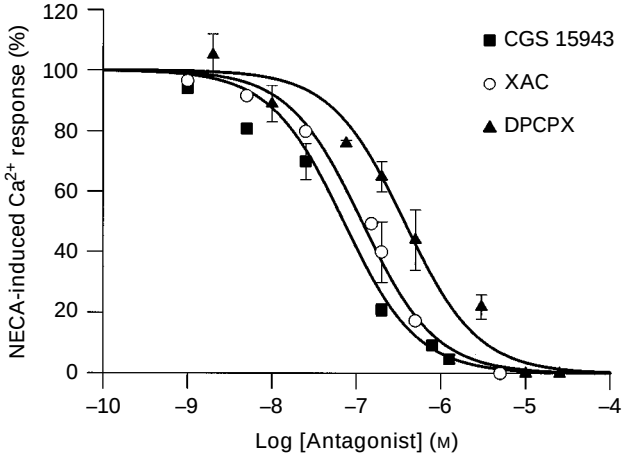

Figure 4 Antagonism of NECA-induced calcium mobilization. Indo-1 loaded cells were preincubated for 3 min with vehicle or with the different reagents before the addition of 50 μM NECA. None of the antgonists was able to induce Ca^{2+} mobilization per se. The calculated pIC_{50} ± s.d. values were: 7.1 ± 0.1 for CGS15943, 6.9 ± 0.1 for XAC and 6.4 ± 0.1 for DPCPX.

lysed by flow cytometry at different periods after the stimulus (Figure 6a). The number of cells that responded to NECA was lower (31%) than those responding to OKT3 (74%). It should be noted that most of the cells responding to NECA after a previous stimulus with OKT3 came from the OKT3-sensitive subpopulation (Figure 6b). To analyse whether the refractoriness of some cells to NECA was linked to a specific stage of the cell cycle, the DNA content was measured in combination with the increase in intracellular calcium. In these assays no differences in behaviour for cells at different stages of the cell cycle were observed (Figure 7). In summary, NECA, at a concentration of 250 μM, led to a cell cycle-independent calcium mobilization in approximately one third of the cells exposed to the compound.

Signal transduction mechanisms involved in the NECA-induced calcium mobilization

A set of experiments was designed to identify the signal transduction pathway involved in the NECA-induced calcium mobilization. For comparative purposes some experiments were performed using OKT3, which leads to the release of calcium from pool I and is inositol(1,4,5)P_3-sensitive. The effect induced by NECA was both pertussis toxin and cholera toxin sensitive, in contrast to OKT3, whose action was cholera toxin sensitive but pertussis toxin insensitive (Figure 8). NECA-induced calcium mobilization was not dependent on increases in cAMP because 10 μM forskolin, which produces a more potent cAMP accumulation than 100 μM NECA (2.7 fold higher), did not modify the calcium levels (data not shown). On the other hand, unlike OKT3, which markedly increased inositol phosphate levels (2.6-fold at a concentration of 1 μg ml^{-1}), NECA up to 250 μM did not induce inositol phosphate production. Depletion of the calcium pool linked to A_{2b} receptors did not modify the calcium response to OKT3 (Figure 9). Moreover, depletion of inositol phosphate-sensitive calcium stores by repeated stimulation with OKT3 did not modify the calcium response to NECA (Figure 9). Furthermore, the effects of NECA and OKT3 were synergistic (Figure 9). These results demonstrate that the pools affected by NECA triggering and by OKT3 triggering are different and that they can occur simultaneously and synergistically in the same cell.

It was then suspected that a pool sensitive to cGMP was involved in the cell response via A_{2b} receptors. To explore this possibility, the level of cGMP was measured in cells incubated with NECA. In fact, cGMP levels did rise in response to NECA. A 3.9 fold increase upon basal levels was found at

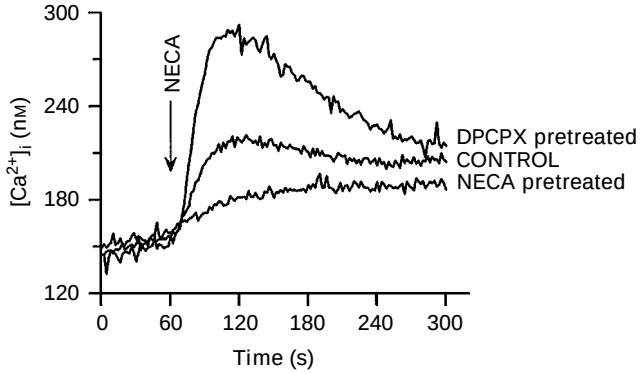

Figure 5 Calcium signal in cells pretreated with NECA or DPCPX. 5×10^5 cells/ml were incubated in the absence or in the presence of 25 μM NECA or 1 μM DPCPX for 40 h at 37°C in complete medium and then washed and loaded with Indo-1. Cell number and viability were unaffected during incubations. The arrow indicates the addition of 250 μM NECA and traces correspond to a representative experiment. Incubation with DPCPX upregulated the response (202±7% of control) while preincubation with NECA downregulated it (28±15% of control).

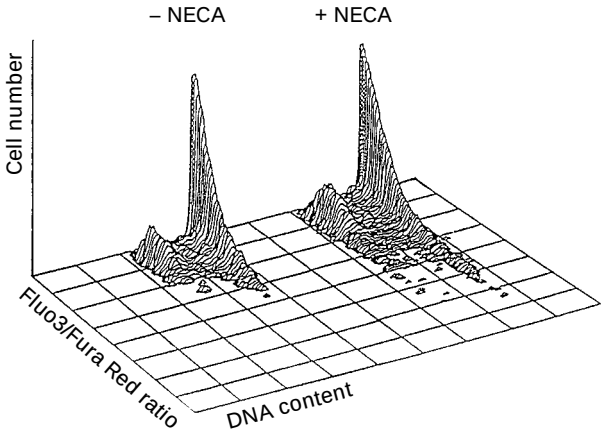

Figure 7 Combined quantification of DNA content and intracellular calcium. Hoechst 33342 was added to Fluo-3/Fura Red loaded cells 30 min before analysis by flow cytofluorimetry, as described in Methods. Tridimensional representations correspond to values of Fluo-3/Fura Red ratio and DNA content for control cells (left) and for cells treated with 250 μM NECA (right).

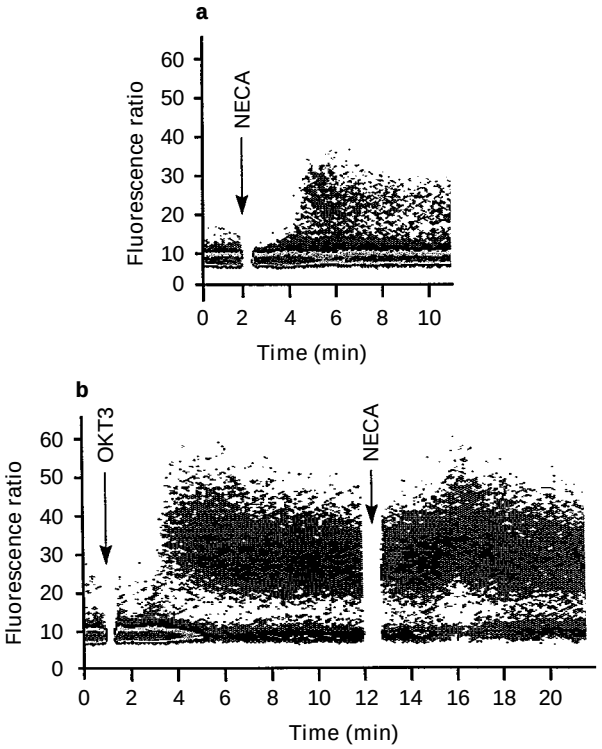

Figure 6 Flow cytometric analysis of Ca^{2+} mobilization induced by NECA and OKT3. Indo 1-loaded cells were stimulated by addition of 250 μM NECA or 1 μg ml^{-1} OKT3 antibody at the time indicated with an arrow and the 395/525 nm fluorescence ratio was analysed as described in Methods. (a) Effect of NECA in unstimulated cells. (b) Effect of NECA in cells previously stimulated via the TCR/CD3 complex.

0.5 μM NECA. This increase was similar to that found at higher NECA concentrations probably due to some degree of downregulation in the response. Interestingly, the inhibitor of guanylate cyclase, LY83583, partially inhibited the calcium signal elicited by NECA but only in the presence of extracellular Ca^{2+} (Figure 10). When EGTA was present in the assays LY83583 did not affect the NECA-induced calcium mobilization. The diffusable analogue of cGMP, 8-Br-cGMP (up to 300 μM), did not modify the intracellular calcium concentration nor affect the NECA-induced calcium mobilization. As nitric oxide is a potent activator of guanylate cyclase (Arnold *et al*., 1977), adenosine and nitric oxide may share a common transduction mechanism. For this reason we tested the ability of a precursor of nitric oxide, sodium nitroprusside, to mobilize calcium. The compound was not able to increase the intracellular calcium concentration even under the conditions described by Clementi *et al*. (1996), nor did it affect the action of NECA on the increase in intracellular calcium concentration. Taken together, these data indicate that stimulation of A_{2b} receptors leads to mobilization of calcium from intracellular stores via an inositol phosphate- and cGMP-independent mechanism.

Discussion

Jurkat cells did not significantly bind [^{3}H]DPCPX or [^{3}H]R-PIA with high affinity (see Results), thus ruling out the expression of A_1 adenosine receptors in these cells. The lack of the A_1 subtype was also established by immunoblotting experiments (data not shown) using a specific antibody whose characterization is described elsewhere (Ciruela *et al*., 1995). In addition, a ligand specific for A_{2a} receptors, [^{3}H]CGS21680, did not bind to either cells or membranes. Therefore, despite the presence of mRNA for the A_{2a} subclass (van der Ploegh *et al*., 1996), the level of expression of these receptors on the cell surface is very low. The specific binding of [^{3}H]NECA to cell membranes and the order of potency of different adenosine analogues as displacers suggest the presence of A_{2b} receptors. The effective displacement of [^{3}H]NECA specific binding by R-PIA reveals the absence of a low affinity NECA-binding protein (adenotin) on the surface of Jurkat cells. Although NECA produced increases in the level of cAMP, the presence of A_3 receptors cannot be ruled out. The calcium response elicited by NECA cannot, however, be mediated by A_3 receptors, as it was antagonized by DPCPX, which is a xanthine-derivative, and the release of intracellular calcium is not produced by the A_3 specific agonist IB-MECA. Taken together, our results indicate that in Jurkat cells there is coupling between A_{2b} adenosine receptors and intracellular calcium channels.

The positive coupling of A_{2b} receptors to adenylate cyclase has been extensively studied in Jurkat cells. The first report by Nordstedt *et al*. (1987) showed that activation of protein kinase C potentiates adenosine receptor-stimulated accumulation of cAMP. In a subsequent study, Kvanta *et al*. (1989)

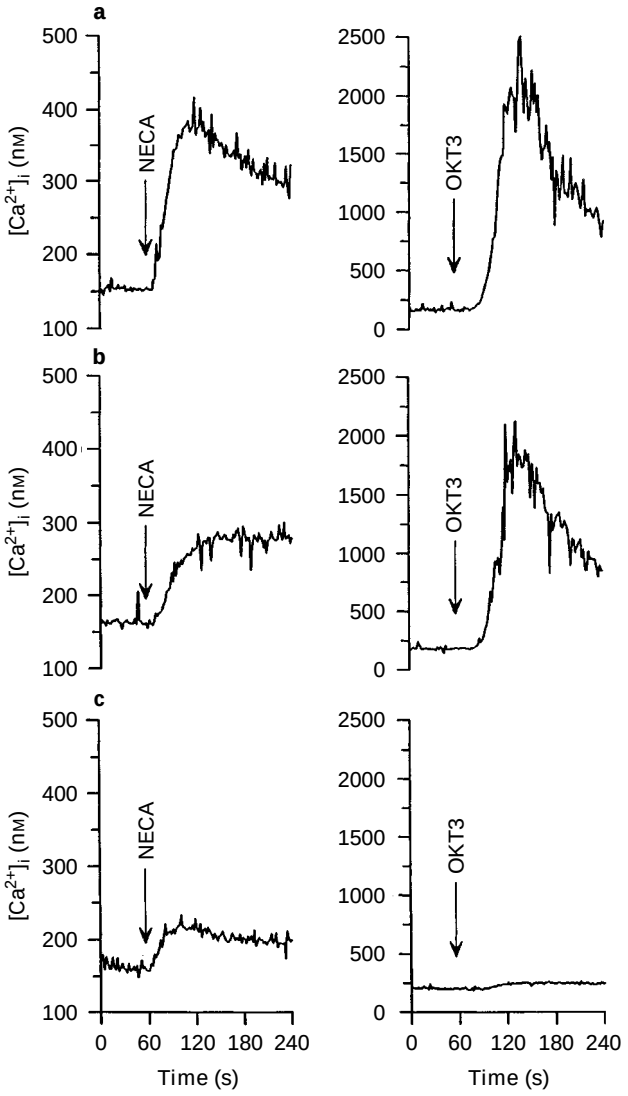

Figure 8 Sensitivity of NECA-induced Ca^{2+} mobilization to cholera and pertussis toxins. Jurkat cells (2×10^6 cells/ml) were incubated with medium (a), with 150 ng ml^{-1} pertussis toxin (b) or with 150 ng ml^{-1} cholera toxin (c) for 5 h at 37°C in complete medium. Different assays were performed with 10% FCS, 0% FCS or 10% heat inactivated FCS in the complete medium to rule out indirect effects of toxins, and no differences were noted. After incubation, cells were washed, loaded with Indo-1 as described in Methods and 250 μM NECA (left) or 1 μg ml^{-1} OKT3 (right) were added to cuvettes. NECA response was inhibited both by pertussis toxin (47 ± 13% inhibition) and cholera toxin (68 ± 10% inhibition), while OKT3 response was only significantly inhibited by cholera toxin as previously described (Gukovskaya 1991). Traces correspond to a representative experiment.

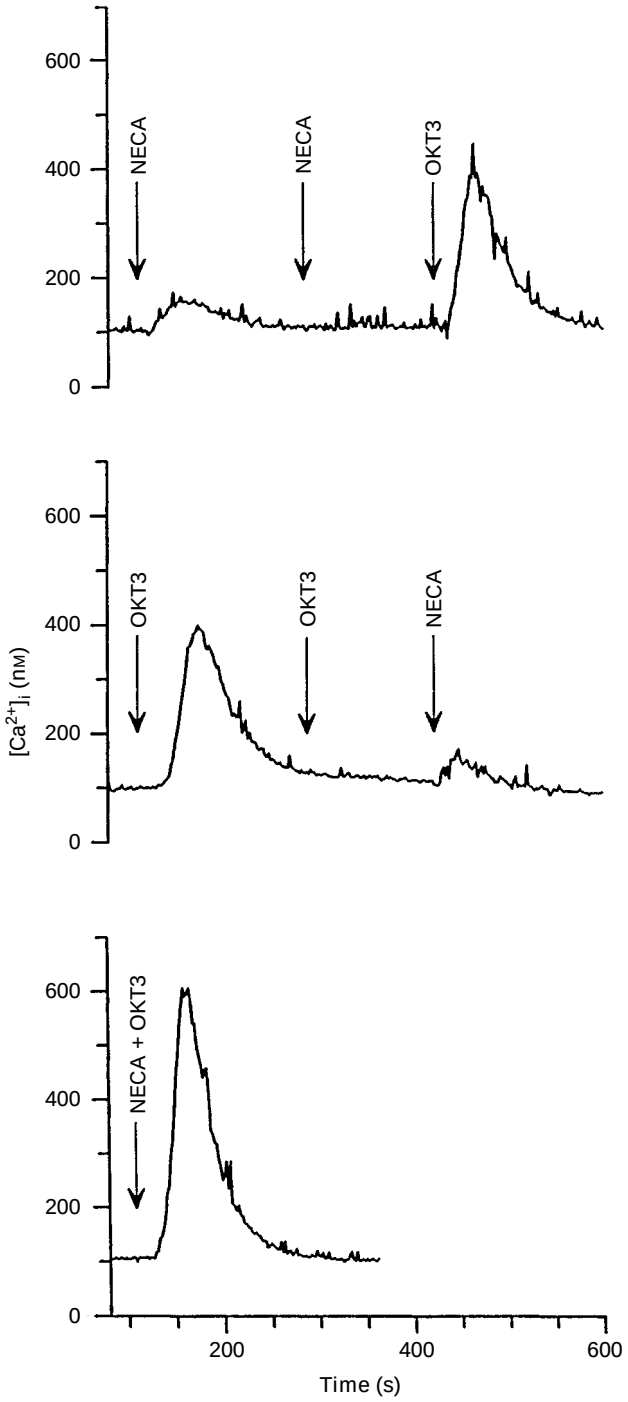

Figure 9 NECA and OKT3 mobilize Ca^{2+} from different intracellular Ca^{2+} pools. Jurkat T cells were loaded with Indo-1 as described in Methods. Addition of NECA (250 μM) and OKT3 (1 μg ml^{-1}) were performed as indicated by arrows; 4 mM EGTA was present in all assays.

demonstrated a functional cross-talk between the T cell receptor and A_{2b} receptors via protein kinase C occurring at the cAMP level. On the basis of these reports, we considered the possibility of a cross-talk between the T cell receptor and A_{2b} receptors operating at the level of intracellular calcium mobilization. It is well known that Jurkat cells specifically activated via the T cell receptor/CD3 complex increase their calcium level via inositol phosphate-dependent mechanisms (Guse *et al*., 1993). However, as indicated in the Results, activation of A_{2b} receptors led to calcium mobilization from inositol phosphate-insensitive stores. Accordingly, the calcium response elicited by agonists of A_{2b} receptors was not abolished by depletion of the stores mobilized by the antibody against the T cell receptor/CD3 complex, which is used to activate the cells *in vitro*. Flow cytometric studies confirmed that calcium mobilization by NECA can occur in cells previously triggered by means of the monoclonal antibody against the TCR/CD3 complex (Figure 6).

The intracellular calcium store linked to A_{2b} receptor-activation may be released from pool III, which is caffeine sensitive (Guse *et al*., 1993). The involvement of pool III is difficult to study because the action of caffeine cannot be easily investigated. Caffeine would lead to the release of calcium from pool III but it would also act as a non-selective antagonist of adenosine receptors. Guse *et al*. (1995) have described that a metabolite of nicotinamide adenine dinucleotide, termed cyclic adenosine diphosphate-ribose, releases Ca^{2+} from caffeine

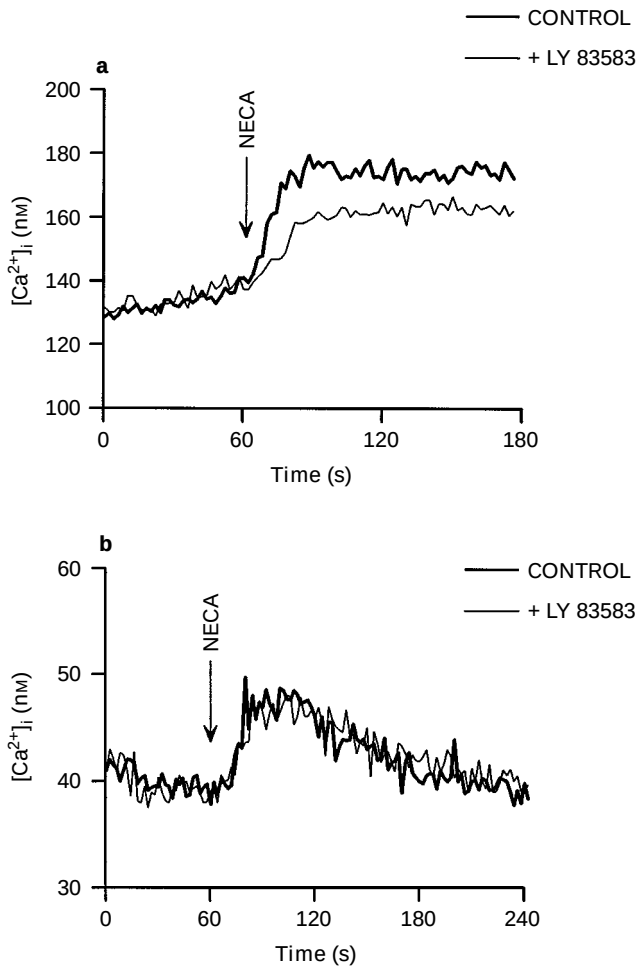

Figure 10 Effect of LY 83583 upon NECA-induced increase of intracellular calcium. Cells were loaded with Fura-2 and preincubated with 100 μM LY 83583 or vehicle for 15 min at 37°C before addition of 100 μM NECA. (a) Data obtained in the absence of EGTA. (b) Data obtained in the presence of EGTA, which was added 60 s prior to the addition of NECA.

sensitive stores in Jurkat cells. The authors also demonstrated the presence of endogenous cyclic adenosine diphosphate-ribose in Jurkat cells, whose formation involves a cGMP-dependent step. Previous reports linked production of cGMP with the activation of cell surface A_1 adenosine receptors in vascular smooth muscle cells (Kurtz, 1987; Broome *et al*., 1994) or A_2 adenosine receptors in pinealocytes (Nikodijevic & Klein, 1989). In Jurkat cells activation of A_{2b} adenosine receptors leads to an increase in cellular levels of both cGMP and Ca^{2+}. However, the lack of effect of diffusible cGMP analogues on calcium mobilization suggests that the mechanism of NECA-induced Ca^{2+} response is not mediated by cGMP. This was confirmed by the lack of effect of sodium nitroprusside, a reagent that generates nitric oxide and, therefore, leads to accumulation of cGMP. The fact that an inhibitor of guanylate cyclase partially inhibited the NECA-induced Ca^{2+} accumulation when the assays were performed in the absence of EGTA but not when the assays were performed in the presence of the Ca^{2+} chelator indicates that cGMP may be necessary but not sufficient for the opening of plasma membrane calcium channels, whereas it does not affect intracellular channels. The reason why we did not find Ca^{2+} responses using diffusible cGMP analogues, whereas Guse *et al*. (1995) demonstrated that cGMP can mobilze Ca^{2+} in permeabilized Jurkat cells,may be due to functional differences derived from the permeabilization procedure. Alternatively the manner in which cGMP production is coupled to the generation of cyclic adenosine diphosphate-ribose may depend on the Jurkat T cell clone.

In contrast to what happens in Jurkat cells, A_{2b} receptors are not directly coupled to intracellular calcium channels in the human erythroleukaemia cell line TIB180. In these cells adenosine analogues only potentiated the calcium signal produced by thrombin and this occurred when extracellular calcium was present (Feoktistov *et al*., 1994). In this case, although the potentiation effect is mimicked by cholera toxin, it is not related to the increase in cAMP elicited by agonists of the A_{2b} receptor. Therefore, A_{2b} receptors, which are coupled to adenylate cyclase via stimulatory G proteins in all cells assayed, can couple to different calcium channels using different signal transduction mechanisms depending upon the cell. The fact that the NECA-induced calcium mobilization is sensitive to both pertussis and cholera toxin reinforces the view that A_{2b} receptors might couple to different G proteins. To deliver the calcium signal, A_{2b} receptors probably interact with a pertussis and cholera sensitive G protein. On the other hand, the results presented provide a novel transduction mechanism for adenosine in T cells linking A_{2b} receptors to calcium stores in an inositol phosphate- and cGMP-independent fashion. The physiological significance of this mechanism may be related to a direct role for adenosine in potentiating signals delivered through the T cell receptor.

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