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Positive Modulation of Intracellular Ca^{2+} Levels by Adenosine A_{2b} Receptors, Prostacyclin, and Prostaglandin E_1 via a Cholera Toxin-Sensitive Mechanism in Human Erythroleukemia Cells

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SUMMARY

Human erythroleukemia (HEL) cells express megakaryocyte/platelet membrane markers and thus have been used as a model for studying platelet membrane receptors and their coupling to cell signaling pathways. Our previous studies, however, indicated that platelets and HEL cells possess different subtypes of adenosine A_2 receptors. Furthermore, we now report that, whereas adenosine inhibits intracellular Ca^{2+} increases in platelets, it potentiates the rise in intracellular Ca^{2+} produced by thrombin, prostaglandin E_1, thapsigargin, and the calcium ionophore A23187 in HEL cells. Stable adenosine analogs potentiated intracellular Ca^{2+} increases with a rank order of potencies of 5′-*N*-ethylcarboxamidoadenosine (NECA) > (*R*)-(−)-N^6-(2-phenylisopropyl)adenosine (*R*-PIA) ≫ CGS 21680, suggesting that this effect is mediated by A_{2b} receptors. EC_{50} values for NECA and *R*-PIA were 0.8 and 42 μM, respectively. NECA (100 μM) potentiated by 2–3-fold the increase in intracellular Ca^{2+} produced by 0.3 unit/ml thrombin. This effect was mimicked by cholera toxin and was shared by other G_s-coupled receptors, such as those activated by the prostacyclin analog iloprost and prostaglandin E_1, indicating the involvement of G_s proteins. Adenosine analogs also increased intracellular cAMP with the same rank order of potencies. The membrane-permeable analog 8-bromo-cAMP, however, had no effect on intracellular Ca^{2+} levels, indicating that the potentiation of intracellular Ca^{2+} increases and the activation of adenylate cyclase are parallel but independent events. The increase in intracellular Ca^{2+} produced by adenosine is due not to an increase in phosphoinositide hydrolysis but, rather, to an increase in calcium influx, and it is lost if cells are studied in the absence of extracellular Ca^{2+}. We conclude, therefore, that adenosine A_{2b} receptors in HEL cells are coupled to G_s proteins and their activation leads to stimulation of adenylate cyclase and, independently, to potentiation of the rise in intracellular Ca^{2+}. We speculate that A_{2b} receptors in HEL cells activate a calcium channel through a cholera toxin-sensitive mechanism that requires an initial increase in intracellular Ca^{2+}.

There is growing evidence that adenosine acts as a modulator of many physiological functions. Adenosine, for example, has been implicated in the regulation of neural, cardiovascular, and metabolic processes. Adenosine receptors also are found on platelets, and their activation leads to accumulation of cAMP, as well as inhibition of the rise in intracellular Ca^{2+} and the platelet aggregation produced by thrombin. The study of platelet adenosine receptors, however, is limited by the impossibility of maintaining these cells *in vitro*. HEL cells are derived from a patient with Hodgkin's disease and erythroleukemia. HEL cells express megakaryocyte/platelet membrane markers (1) and, for this reason, have been used as a model system for studying membrane receptors (2, 3) and signal transduction processes (4) as they might relate to platelet/megakaryocyte function.

Our initial studies demonstrated that the activation of adenosine receptors in both platelets and HEL cells results in accumulation of cAMP, but the orders of potencies of agonists and antagonists differ significantly between these cell types (5). These results suggest that platelets and HEL cells may possess different subtypes of adenosine A_2 receptors. Furthermore, whereas adenosine and prostacyclin inhibit the rise in free intracellular Ca^{2+} produced by thrombin in platelets (6), these two agents potentiate the rise in intracellular Ca^{2+} produced by thrombin in HEL cells. The purpose of the present studies was to investigate the intracellular signaling pathways

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ABBREVIATIONS: HEL, human erythroleukemia; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; NECA, 5′-*N*-ethylcarboxamidoadenosine; (*R*)-PIA, (*R*)-(−)-N^6-(2-phenylisopropyl)adenosine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PKA, protein kinase A; PGE_1, prostaglandin E_1; PKI(5–24), protein kinase inhibitor 5–24; 8-Br-cAMP, 8-bromo-cAMP.

involved in the modulation of intracellular Ca^{2+} by adenosine in HEL cells.

Experimental Procedures

Cells. HEL cells (TIB 180) were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in suspension culture, at a density between 3 and 9 × 10^5 cells/ml, by dilution with RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 10% (v/v) newborn calf serum, and 2 mM glutamine. Cells were kept under a humidified atmosphere of air and 5% CO_2 at 37°.

Measurement of intracellular calcium. Cytosolic free calcium concentrations were determined by the fluorescent dye technique. HEL cells (2 × 10^6 cells/ml) were loaded with 1 μM fura-2/acetoxymethyl ester in a buffer containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2P0_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 g/liter D-glucose, 10 mM HEPES-NaOH, pH 7.4, and 0.35% bovine serum albumin (buffer A). After incubation for 30 min, cells were washed to remove excess fura-2/acetoxymethyl ester and were resuspended in buffer A at a concentration of 2 × 10^6 cells/ml. Just before each measurement, 100 μl of cell suspension were diluted to 2 ml in albumin-free buffer A. The fluorescence of HEL cells in suspension was monitored at an emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm. Maximal fluorescence was determined by addition of 20 μl of 0.4% digitonin. Minimal fluorescence was determined by addition of 40 μl of 1 M EGTA. The intracellular calcium concentration was calculated by using previously described formulas (7), assuming a K_d of 224 nM for binding of Ca^{2+} by fura-2. Fluorescence was measured with a Fluorolog-2 spectrofluorimeter (SPEX Industries, Inc., Edison, NJ).

Measurement of cAMP. Before each experiment, cells were harvested, washed by centrifugation (100 × *g* for 10 min), and resuspended in RPMI 1640 medium without phenol red pH indicator, to a concentration of 10^7 cells/ml. HEL cells (2 × 10^6/tube) were preincubated for 2 min at 37° in a total volume of 200 μl of RPMI 1640 medium containing the cAMP phosphodiesterase inhibitor papaverine (0.1 mM). In ancillary studies we determined that the increase in cAMP produced by adenosine was not different if HEL cells were incubated in RPMI 1640 medium or in buffer A. cAMP accumulation in response to adenosine agonist was measured by the addition of the agonist (2 μl) to the cell suspension. Cells were then mixed with a vortex mixer and the incubation was allowed to proceed for 2 min at 37°. The reaction was stopped by addition of 50 μl of 25% trichloroacetic acid. Trichloroacetic acid-treated extracts were washed five times with 10 volumes of water-saturated ether. cAMP concentrations were determined by competition binding of tritium-labeled cAMP to a protein (derived from bovine muscle) that has high specificity for cAMP (8) (cAMP assay kit, TRK.432; Amersham, Arlington Heights, IL).

Measurement of 8-Br-cAMP incorporation into HEL cells. HEL cells were incubated for 5 min at room temperature in buffer A with increasing concentrations of 8-Br-cAMP. For each concentration of 8-Br-cAMP a paired sample was incubated with an identical concentration of cAMP, as a nonpermeable control. After centrifugation at 11,500 × *g* for 1 min at 4°, cells were resuspended in buffer A to a total volume of 200 μl. In ancillary studies we found that HEL cells remain intact after this centrifugation, as assessed by the trypan blue method. Cell suspensions were then mixed with 50 μl of 25% trichloroacetic acid, and extracts were washed five times with diethyl ether. 8-Br-cAMP and cAMP concentrations were measured using the competition binding method described above. Individual calibration curves were constructed for 8-Br-cAMP and for cAMP. Incorporation of 8-Br-cAMP into HEL cells was determined as the difference between 8-Br-cAMP and cAMP concentrations in paired samples.

Measurement of PKA activity. HEL cells were resuspended at 3 × 10^7 cells/ml in ice-cold homogenization buffer containing 150 mM KCl, 10 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 20 mM MOPS, pH 6.6. Cells were homogenized using a Teflon pestle in a glass homogenizer tube. After 3-fold dilution with the same ice-cold buffer, homogenates were assayed immediately by a modification of the procedure proposed by Witt and Roskoski (9). In brief, the final incubation mixture contained 20 mM MOPS, pH 6.6, 1 mM dithiothreitol, 1 mM EGTA, 10 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP (0.5 μCi), 1 mM isobutylmethylxanthine, 0.5 mg/ml histone IIAS, and 0.05% (v/v) Triton X-100. The reaction was initiated by addition of 25 μl of diluted homogenate (10^7 cells/ml) to 25 μl of the other components. The incubation was carried out at 22° for 5 min and was terminated by the blotting of 25 μl of the mixture onto Whatman phosphocellulose P81 filter paper (2 × 2 cm). Filters were washed twice with 0.5% (v/v) phosphoric acid for 20 min, using a magnetic stirrer, and once with ethanol. After drying in air, the radioactivity absorbed onto the phosphocellulose was measured by liquid scintillation counting. The activity of PKA was calculated as the difference between total protein kinase activity and activity in the presence of 1 μM PKI(5–24), a synthetic protein kinase inhibitor (LC Laboratories, Woburn, MA). In ancillary studies we found that 1 μM PKI(5–24) completely reversed the activation of PKA produced by 10 μM cAMP, in agreement with previously published data (10).

Measurement of phosphoinositide hydrolysis. HEL cells were resuspended at 10^6 cells/ml in inositol-free RPMI 1640 medium and were incubated for 20 hr with *myo*-[^{3}H]inositol (5 μCi/ml; DuPont NEN Research Products, Boston, MA). The HEL cells were then washed twice and resuspended in buffer A, at a cell concentration of 5 × 10^7 cells/ml. LiCl was included in the incubation buffer at a final concentration of 20 mM when noted. Aliquots of cells (197 μl) were prewarmed at 37° for 10 min before addition of stimulants (3 μl) or buffer A with an equivalent concentration of dimethylsulfoxide. Reactions were terminated by addition of 25 μl of ice-cold 100% trichloroacetic acid, and samples were prepared for high performance liquid chromatographic analysis as described previously (11). Briefly, after trichloroacetic acid precipitation, supernatants were washed six times with diethyl ether and then neutralized with 1 M Tris, pH 8.0. Before chromatographic analysis, a mixture of unlabeled ATP, ADP, and AMP was added to each sample for UV detection of peak elution, and the samples were passed through a 0.45-μm HV filter (Millipore Corp., Bedford, MA). After an initial 5-min wash with water to elute free inositol, the [^{3}H]inositol phosphates (10^7 cells/sample) were separated by high performance liquid chromatography on a Whatman Partisil-SAX column (0.46 × 25 cm), using a linear gradient from 0 to 0.8 M ammonium phosphate (pH 3.8, adjusted with phosphoric acid) over 75 min. Peaks of radioactivity corresponding to the various [^{3}H]inositol phosphates were monitored with an on-line flow detector (Radiomatic Instruments, Meridian, CT), using Tru-Count scintillation fluid (IN-US Corp., Fairfield, NJ) (3:1 ratio). The radioactive peaks were identified in relation to the elution of the UV-absorbing standards.

Drugs. DPCPX, NECA, (*R*)-PIA, and CGS 21680 [2-[*p*-(carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine hydrochloride] were purchased from Research Biochemicals, Inc. (Natick, MA). PGE_1 was purchased from Cayman Chemical Co. Inc. (Ann Arbor, MI). Papaverine and 8-Br-cAMP were obtained from Sigma Chemical Co. (St. Louis, MO). Iloprost, a stable analog of prostacyclin, was a gift from Schering AG (Berlin, Germany) and Berlex Laboratories, Inc. (Wayne, NJ). Forskolin was purchased from Calbiochem Corp. (La Jolla, CA). Thapsigargin and the PKA inhitor PKI(5–24) were obtained from LC Laboratories.

Data analysis. Calculation of EC_{50} values from dose-response curves and data analysis by nonlinear regression were performed using ALLFIT 2.6 software (Laboratory of Theoretical and Physical Biology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD) and InPlot 4.0 software (GraphPAD Software, San Diego, CA), on a microcomputer. Statistical analysis was performed using InStat 2.0 software (GraphPAD Software). Unpaired *t* tests were used for single comparisons. Analysis of variance was used for multiple comparisons. The criterion for significance was $p < 0.05$. Results are presented as mean ± standard error.

Results

Effect of prostacyclin and PGE_1 on basal intracellular calcium levels. Both PGE_1 and the stable prostacyclin analog iloprost produced a dose-dependent increase in intracellular calcium content (Fig. 1), with PGE_1 being more potent than iloprost. Both prostaglandins also increased cAMP content in the cells (Fig. 1). PGE_1 increased the levels of cAMP and Ca^{2+} in approximately the same range of concentrations. In contrast, there was a larger discrepancy in potencies for iloprost-induced rises in cAMP and intracellular Ca^{2+}. We also studied the effects of cholera toxin and pertussis toxin, to determine the role of G proteins in the actions of PGE_1 and iloprost. Preincubation of HEL cells with pertussis toxin (500 ng/ml for 24 hr) had no effect on basal intracellular Ca^{2+} levels. Pertussis toxin, however, significantly prevented the rise in intracellular calcium produced by PGE_1 but not the increase in intracellular calcium produced by iloprost (Fig. 2); PGE_1 (1 μM) increased intracellular calcium by 129 ± 5 nM in control cells but by 17 ± 5 nM in pertussis toxin-treated cells (six experiments, $p < 0.001$ by t test). Preincubation of HEL cells with cholera toxin (100 ng/ml for 24 hr) had no effect on basal intracellular calcium or on the increase in intracellular calcium produced by PGE_1. Cholera toxin reduced the increase in intracellular calcium produced by iloprost (Fig. 2), but this effect did not reach statistical significance; iloprost (1 μM) increased intracellular calcium by 42 ± 5 nM in control cells and by 26 ± 2 nM in cholera toxin-treated cells (four experiments, $p > 0.05$ by t test).

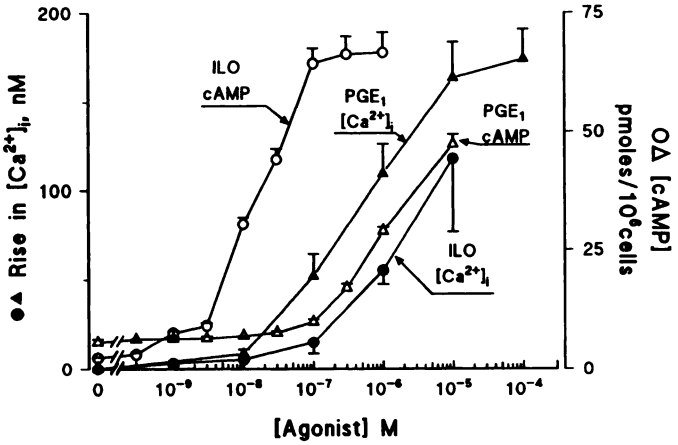

Fig. 1. Effect of increasing concentrations of PGE_1 (*triangles*) and iloprost (*ILO*) (*circles*) on the levels of free intracellular Ca^{2+} (*left y-axis*) (*closed symbols*) and cAMP (*right y-axis*) (*open symbols*) in HEL cells.

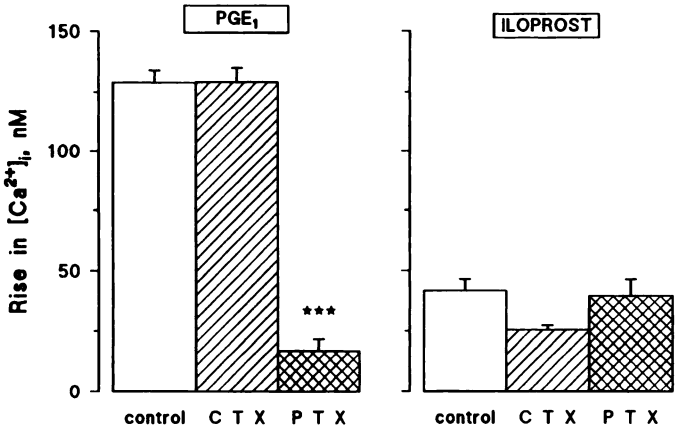

Fig. 2. Rise in free intracellular Ca^{2+} levels produced by PGE_1 (1 μM) (*left*) and iloprost (1 μM) (*right*) in control HEL cells (*control*) and in cells incubated with 100 ng/ml cholera toxin (*CTX*) or 500 ng/ml pertussis toxin (*PTX*) for 24 hr. ***, Statistical differences, compared with control ($p < 0.001$).

Effect of adenosine receptor activation on intracellular calcium levels. In contrast to the effects of PGE_1 and iloprost, adenosine had no effect on basal intracellular Ca^{2+} levels at concentrations of up to 1 mM. On the other hand, adenosine both potentiated the maximal increase in intracellular calcium produced by thrombin and accelerated its rate of increase (Fig. 3). The stable adenosine analog NECA potentiated the increase in intracellular calcium to a similar degree throughout the effective concentrations of thrombin (Fig. 3). Subsequent studies were done by adding adenosine or adenosine analogs 30 sec before 0.3 unit/ml thrombin. In ancillary studies, we found that adenosine and its analogs also potentiated the intracellular Ca^{2+} increase if they were added at the peak of the response to thrombin (data not shown).

We thought it important to determine whether adenosine also potentiated the increase in Ca^{2+} produced by other receptor-mediated or non-receptor-mediated mechanisms. In the presence of extracellular Ca^{2+}, NECA (100 μM) also potentiated the increases in intracellular Ca^{2+} produced by PGE_1 (1 μM) (from 119 ± 29 to 227 ± 35 nM, three experiments, $p < 0.05$ by t test), the calcium ionophore A23187 (10 nM) (from 246 ± 12 to 443 ± 31 nM, five experiments, $p < 0.001$ by t test), and the microsomal calcium ATPase inhibitor thapsigargin (12) (10 nM) (from 559 ± 27 to 671 ± 20 nM, five experiments, $p < 0.05$ by t test).

Stable adenosine analogs were used to study the adenosine receptor subtype involved in this action. The mixed A_{2a}/A_{2b} adenosine agonist NECA was 50 times more potent than the selective A_1 adenosine agonist (R)-PIA (EC_{50}, 0.8 ± 0.4 and 41.8 ± 16.2 μM, respectively; nine experiments) in potentiating thrombin-induced Ca^{2+} increases (Fig. 4). The selective A_{2a} agonist CGS 21680 had no effect on thrombin-induced Ca^{2+} rises at concentrations of up to 100 μM (data not shown). The adenosine receptor antagonist DPCPX did not prevent adenosine actions at a concentration of 5 nM, which selectively blocks A_1 receptors (13). A higher concentration of DPCPX (100 μM), which blocks both A_1 and A_2 receptors, had no direct effect on the thrombin-induced rise in intracellular calcium but completely abolished its potentiation by adenosine; adenosine (100 μM) potentiated the increase in intracellular Ca^{2+} produced by thrombin (from 162 ± 24 to 359 ± 50 nM, four experiments, $p < 0.01$) in the absence of DPCPX but had no effect (from 170 ± 26 to 189 ± 24 nM) in the presence of 100 μM DPCPX. The rank order of potencies for agonists and the response to DPCPX, therefore, suggest that the adenosine receptor involved in modulation of intracellular calcium belongs to the A_{2b} subtype. The same receptor subtype has been implicated in the modulation of cAMP in this cell type (5).

We recently found that differentiation of HEL cells leads to the loss of adenosine-induced stimulation of adenylate cyclase, whereas iloprost- and forskolin-induced stimulation of adenylate cyclase remains virtually intact (5). We therefore determined the effect of differentiation of HEL cells on the regulation of Ca^{2+} levels by adenosine. For this purpose HEL cells were exposed to 1.6 μM 12-*O*-tetradecanoylphorbol-13-acetate for 3 days. Adenosine had no effect on the thrombin-induced

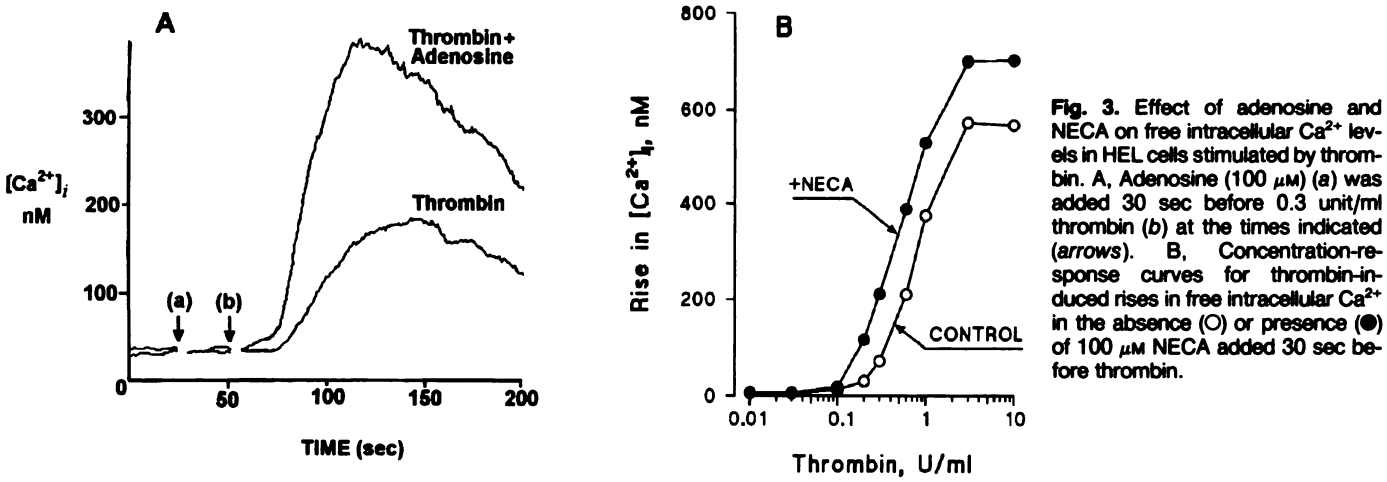

Fig. 3. Effect of adenosine and NECA on free intracellular Ca^{2+} levels in HEL cells stimulated by thrombin. A, Adenosine (100 μM) (*a*) was added 30 sec before 0.3 unit/ml thrombin (*b*) at the times indicated (*arrows*). B, Concentration-response curves for thrombin-induced rises in free intracellular Ca^{2+} in the absence (○) or presence (●) of 100 μM NECA added 30 sec before thrombin.

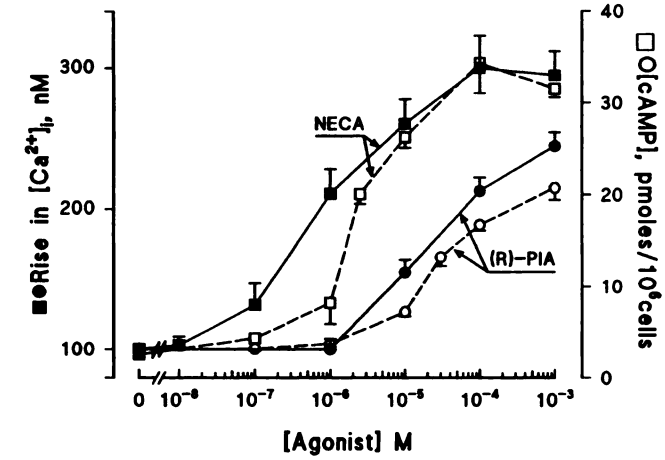

Fig. 4. Effect of increasing concentrations of the adenosine receptor agonists NECA (*squares*) and (*R*)-PIA (*circles*) on free intracellular Ca^{2+} levels (*left y-axis*) (*closed symbols*) and cAMP accumulation (*right y-axis*) (*open symbols*) in HEL cells stimulated by thrombin. Agonists were added 30 sec before 0.3 unit/ml thrombin.

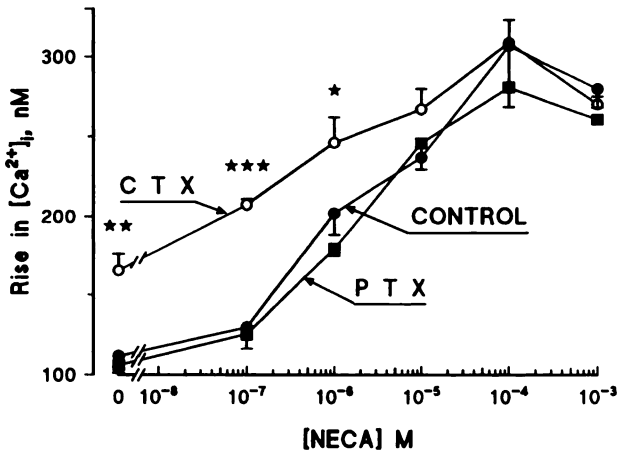

Fig. 5. Effect of increasing concentrations of NECA on free intracellular Ca^{2+} levels in HEL cells stimulated by thrombin. Cells were studied after incubation with buffer (*CONTROL*) (●), 500 ng/ml pertussis toxin (*PTX*) (■), or 100 ng/ml cholera toxin (*CTX*) (○) for 24 hr. NECA was added 30 sec before 0.3 unit/ml thrombin. Statistical differences, compared with control: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

rise in Ca^{2+} in differentiated cells, at concentrations of up to 1 mM. On the other hand, iloprost (1 μM) increased the thrombin-induced rise in intracellular Ca^{2+} by similar magnitudes in differentiated (from 50 ± 2 to 123 ± 6 nM, three experiments, $p < 0.001$ by *t* test) and undifferentiated (from 88 ± 4 to 196 ± 4 nM, three experiments, $p < 0.001$) cells.

Role of G proteins in adenosine actions. To determine the potential role of G proteins in adenosine actions, cholera toxin was used as a way to increase the basal activity of G_s proteins and pertussis toxin was used as a way to block the G_i family of G proteins. Pretreatment of HEL cells for 24 hr with 500 ng/ml pertussis toxin had no effect on basal intracellular Ca^{2+} levels, on the increase in intracellular calcium levels produced by thrombin alone, or on the potentiation of the increase in intracellular Ca^{2+} levels produced by NECA (Fig. 5). Pretreatment of HEL cells with 100 ng/ml cholera toxin for 24 hr potentiated the Ca^{2+} response to thrombin even in the absence of NECA. Addition of NECA produced an additional increase in intracellular Ca^{2+} levels, but the maximal effect was similar to that observed in control cells or in pertussis toxin-treated cells (Fig. 5), suggesting that the effects of cholera toxin and NECA were not additive but shared the same mechanism.

We then determined whether the potentiation of the thrombin-induced rise in intracellular Ca^{2+} was unique to adenosine receptor activation or was shared by other receptors coupled to G_s proteins. We therefore examined whether prostacyclin and PGE_1 would also modulate the thrombin-induced rise in intracellular Ca^{2+}, if studied under conditions that would not alter base-line intracellular Ca^{2+} levels. For this purpose we used a low concentration of iloprost (100 nM) that, although greatly increasing cAMP (Fig. 1), had virtually no effect on basal intracellular calcium levels (from 31 ± 2 to 37 ± 7 nM, $p > 0.05$). Iloprost greatly potentiated the increase in intracellular Ca^{2+} produced by 0.3 unit/ml thrombin at these relatively low concentrations (Fig. 6); thrombin increased intracellular Ca^{2+} by 56 ± 7 nM in the absence and by 149 ± 17 nM in the presence of iloprost (five experiments, $p < 0.001$). Likewise, 1 μM PGE_1 had no significant effect on basal intracellular Ca^{2+} levels in pertussis toxin-treated cells (from 43 ± 2 to 52 ± 4 nM, 24 experiments, $p > 0.05$) but greatly potentiated the increase in intracellular Ca^{2+} produced by 0.3 unit/ml thrombin (Fig. 6); thrombin increased intracellular Ca^{2+} by 89 ± 8 nM in the absence and by 241 ± 16 nM in the presence of PGE_1 in pertussis toxin-treated cells (four experiments, $p < 0.001$).

Role of cAMP in adenosine actions. It could be postulated

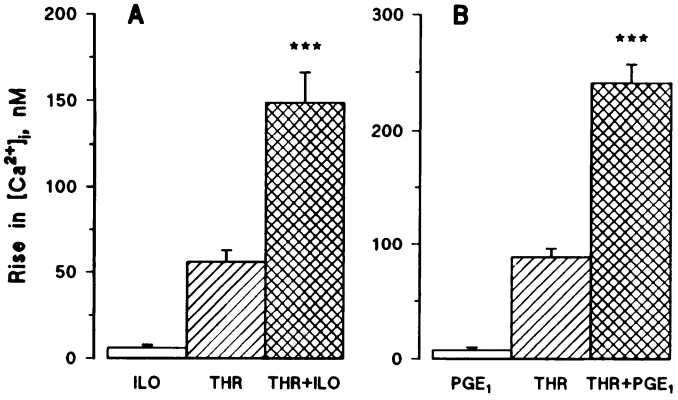

Fig. 6. Potentiation of thrombin-induced rise in free intracellular Ca^{2+} levels by iloprost (A) and PGE_1 (B) (pertussis-treated cells) in HEL cells. A, Increase in free intracellular Ca^{2+} levels produced by iloprost (*ILO*) (100 nM), thrombin (*THR*) (0.3 unit/ml), and iloprost added 30 sec before thrombin (*THR+ILO*). B, Increase in free intracellular Ca^{2+} levels produced by PGE_1 (*PGE_1*) (1 μM), thrombin (*THR*) (0.3 unit/ml), and PGE_1 added 30 sec before thrombin (*THR+PGE_1*), in cells incubated with 500 ng/ml pertussis toxin for 24 hr. ***, Statistical differences, compared with the effects of thrombin alone ($p < 0.001$).

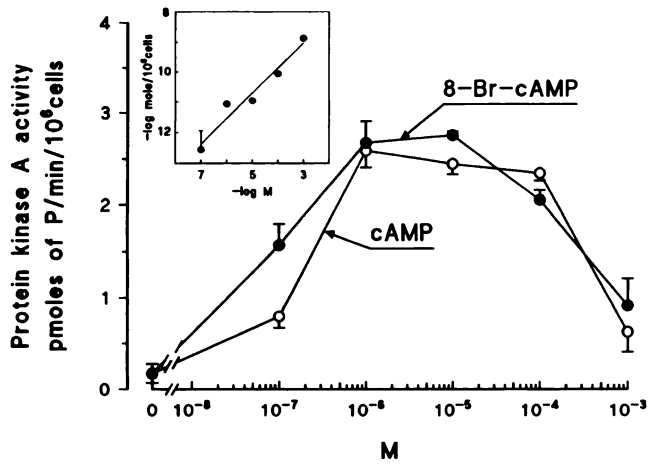

Fig. 7. Effect of cAMP and 8-Br-cAMP on PKA activity in homogenates of HEL cells. *Inset*, incorporation of 8-Br-cAMP into HEL cells (*y-axis*), in relation to its concentration in the incubation medium (*x-axis*) (three experiments). See Experimental Procedures for details.

that adenosine analogs potentiate thrombin-induced rises in intracellular Ca^{2+} by increasing cAMP. If this were the case, then the permeable cAMP analog 8-Br-cAMP should reproduce the effects of adenosine analogs on intracellular Ca^{2+}. To test this hypothesis we first confirmed that 8-Br-cAMP activated PKA activity in HEL cells. A dose-dependent increase in PKA activity was observed at concentration of 8-Br-cAMP between 100 nM and 10 μM (Fig. 7). Higher concentrations of 8-Br-cAMP produced a reversal of this activation; PKA activity returned to nearly basal levels with 10^{-3} M 8-Br-cAMP. This biphasic reponse was identical to that found with cAMP (Fig. 7).

We also confirmed that, under the conditions of our studies, 8-Br-cAMP penetrated into HEL cells. After a 5-min incubation at room temperature, HEL cells incorporated 8-Br-cAMP in a linear relationship with the extracellular concentration ($y = 0.84 \cdot x + 6.5$; $r = 0.97$, $p < 0.01$, three experiments, where x is the negative log of the extracellular concentration of 8-Br-cAMP in the incubation medium and y is the negative log of the intracellular concentration of 8-Br-cAMP in HEL cells) (Fig. 7, *inset*). Incubation of HEL cells with 10 μM 8-Br-cAMP, for example, would increase the intracellular concentrations of this cAMP analog to 50 pmol/10^6 cells. This increase is comparable to the rise in cAMP produced by 100 μM NECA (30–35 pmol/10^6 cells) (Fig. 4). However, 8-Br-cAMP had no effect on the thrombin-induced rise in intracellular Ca^{2+} (Table 1). This was true over a wide range of concentrations (100 nM to 1 mM), including those found to activate PKA. Taken together, therefore, these results suggest that the increase in cAMP and the potentiation of the intracellular calcium increase are parallel, rather than causally linked, events.

Effect of adenosine receptor activation on mobilization and influx of calcium. To investigate the mechanism by which adenosine potentiates the thrombin-mediated rise in intracellular Ca^{2+}, we determined the effect of adenosine on phosphoinositide hydrolysis, the primary pathway stimulating mobilization of internal stores of Ca^{2+}. Thrombin (0.3 unit/ml) increased the formation of inositol trisphosphate, as reported previously (14). NECA (100 μM) did not affect basal or thrombin-mediated inositol trisphosphate formation after 15 or 60 sec of stimulation (Fig. 8A). Thrombin (0.3 unit/ml) stimulated inositol monophosphate accumulation in HEL cells incubated for 15 min in the presence of 20 mM LiCl. This was unaffected by NECA (Fig. 8B).

We used two independent approaches in an attempt to determine whether adenosine-mediated potentiation of intracellular Ca^{2+} increases could be explained by an augmentation in the influx of calcium. First, we determined the effects of adenosine on Mn^{2+}-induced quenching of the fura-2 signal. The two cations share the same channels for entry into cells. At an excitation wavelength of 360 nm and an emission wavelength of 500 nm, fluorescence is selectively quenched by influx of Mn^{2+} and is unaltered by changes in Ca^{2+} (15). Mn^{2+} was added just before each measurement, to a final concentration of 100 μM. As shown in Fig. 9, thrombin increased Mn^{2+}-mediated fluorescence quenching. This quenching was enhanced in the presence of 100 μM NECA, suggesting that calcium influx contributes to the adenosine-mediated rise in intracellular Ca^{2+}. Second, if adenosine increases intracellular Ca^{2+} levels by promoting calcium influx, then this effect would be blunted by acutely reducing the extracellular calcium concentration. For this purpose cells (2×10^6 cells/ml) were kept in buffer containing 1 mM $CaCl_2$, to avoid depletion of intracellular stores. Just before each measurement, 100 μl of cell suspension were diluted to 2 ml in calcium-free buffer (final extracellular Ca^{2+} concentration, 50 μM). Residual extracellular calcium was chelated by addition of 10 μl of EGTA, pH 7.4 (final concentration,

TABLE 1

Effect of 8-Br-cAMP on the increase in intracellular Ca^{2+} produced by thrombin

Cell were preincubated with buffer (control) or increasing concentrations of 8-Br-cAMP for 5 min at room temperature before the addition of 0.3 unit/ml thrombin. Data are expressed as mean ± standard error of three experiments.

Intracellular Ca^{2+} increase					
Control*	8-Br-cAMP				
	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M
	% of control				
100 (166 ± 6 nM)	80 ± 3	92 ± 15	104 ± 5	96 ± 7	100 ± 14

* Thrombin (0.3 unit/ml) alone.

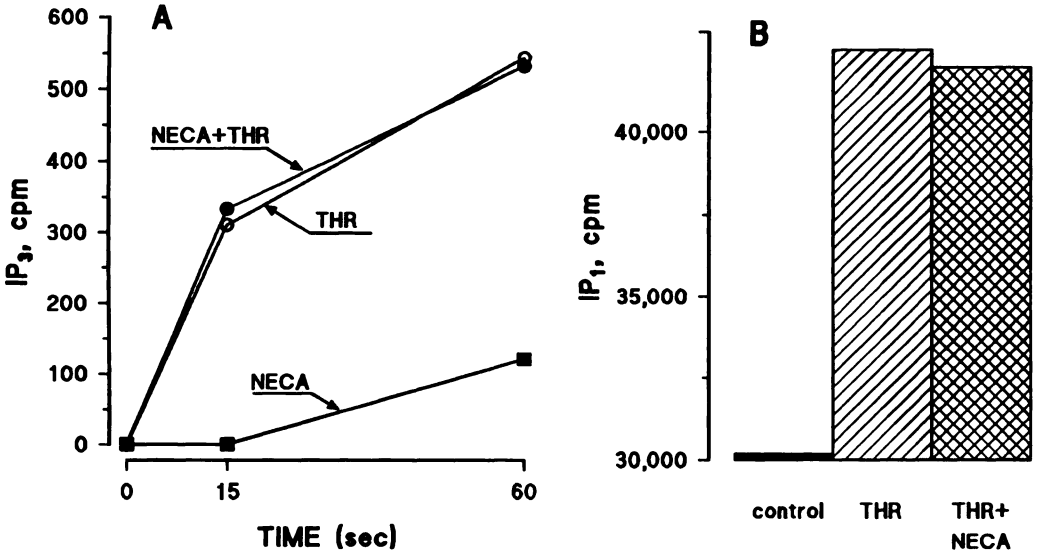

Fig. 8. Effect of NECA on the formation of inositol phosphates in HEL cells stimulated by thrombin. A, Time course of the formation of inositol trisphosphate (IP_3) produced by 0.3 unit/ml thrombin alone (*THR*) (○), 100 μM NECA alone (*NECA*) (■), and NECA added 30 sec before thrombin (*NECA+THR*) (●). B, Accumulation of inositol monophosphate (IP_1) in unstimulated cells (*control*), in cells stimulated with 0.3 unit/ml thrombin (*THR*), and in cells stimulated with 100 μM NECA added 30 sec before thrombin (*THR+NECA*). HEL cells were incubated for 15 min in the presence of 20 mM LiCl. Data are expressed as the average of two independent experiments, which produced similar results.

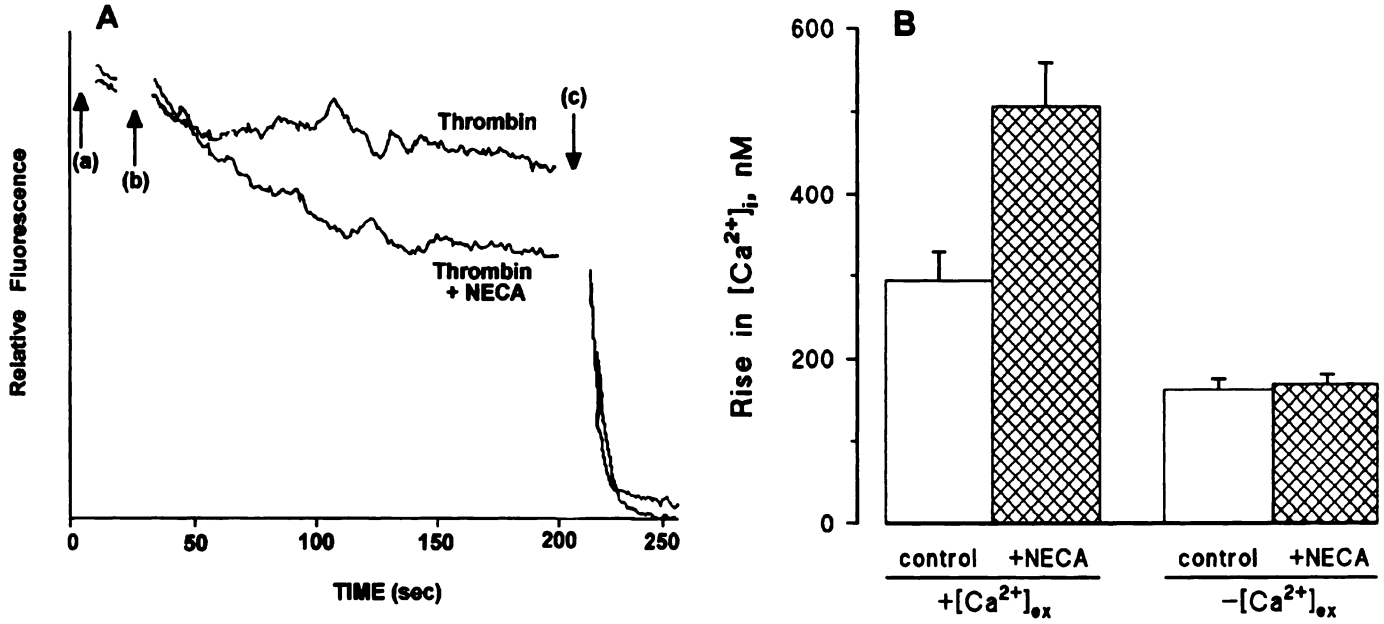

Fig. 9. Effect of NECA on Ca^{2+} influx and mobilization from internal stores. A, Effect on calcium influx of 0.3 unit/ml thrombin (*Thrombin*) and of 100 μM NECA added 30 sec before thrombin (*Thrombin + NECA*). *Arrows*, addition of NECA (*a*), thrombin (*b*), and digitonin (*c*). Calcium influx was determined by the decrease in relative fluorescence due to Mn^{2+}-induced quenching of fura-2 fluorescence. Manganese was added 45 sec before thrombin, to a final concentration of 100 μM. Cells were maintained in 1 mM $CaCl_2$ buffer and were resuspended immediately before each measurement in calcium-free buffer, to reach a final concentration of 50 μM $CaCl_2$. B, Effect of 100 μM NECA on the thrombin-induced rise in intracellular Ca^{2+} in the presence ($+[Ca^{2+}]_{ex}$) or absence ($-[Ca^{2+}]_{ex}$) of extracellular Ca^{2+} in HEL cells. Experiments were performed as described above. EGTA instead of $MnCl_2$ was added 45 sec before thrombin, to a final concentration of 1 mM.

1 mM). As seen in Fig. 9, the potentiation by NECA of the thrombin-mediated rise in intracellular Ca^{2+} was not observed in the absence of extracellular calcium.

Discussion

Adenosine is generally considered an inhibitory autacoid whose role is to counteract excessive metabolic demands on the organism. Activation of adenosine A_1 and A_{2a} receptors, for example, leads to inhibition of neural function, inhibition of the release of a variety of neurotransmitters, inhibition of sinus node automaticity and cardiac conductivity, and vascular relaxation, among other actions (16).

In agreement with this inhibitory role, adenosine prevents the rise in intracellular Ca^{2+} and the platelet aggregation produced by thrombin in human platelets (6). We found, however, that adenosine potentiates rather than inhibits the rise in intracellular Ca^{2+} produced by thrombin in HEL cells (Fig. 3). Adenosine also potentiates the rise in intracellular Ca^{2+} produced by PGE_1, thapsigargin, and the calcium ionophore A23187, indicating that the positive modulatory effects of adenosine on intracellular Ca^{2+} levels require an initial increase in intracellular Ca^{2+}, whether this increase is produced by independent receptor-mediated or non-receptor-mediated events.

We have previously characterized the presence of A_{2b} receptors in HEL cells and their coupling to adenylate cyclase (5).

Adenosine analogs increased cAMP and potentiated intracellular Ca^{2+} increases with similar potencies, suggesting that both effects are mediated by A_{2b} receptors and may share intracellular signaling pathways. Indeed, adenosine potentiated intracellular Ca^{2+} increases through a cholera toxin-sensitive, pertussis toxin-insensitive mechanism (Fig. 5), indicating the involvement of G_s proteins in this process. Furthermore, the positive modulation of intracellular Ca^{2+} is not limited to adenosine receptor activation but is shared by other receptors, i.e., iloprost and PGE_1, that have in common their coupling to G_s proteins.

Given these initial results, it could be postulated that the positive modulation of intracellular Ca^{2+} levels by adenosine is a consequence of the increase in cAMP. However, the membrane-permeable analog 8-Br-cAMP did not replicate the positive modulatory effects of adenosine on intracellular Ca^{2+} levels at concentrations shown to activate PKA. We conclude, therefore, that activation of adenosine receptors, via coupling to G_s proteins, produces parallel and independent increases in intracellular Ca^{2+} and in cAMP.

The potentiation of intracellular Ca^{2+} increases produced by adenosine was observed only in the presence of extracellular Ca^{2+} and was not due to inositol trisphosphate-mediated mobilization of internal calcium. Adenosine increased manganese influx, which presumably uses the same channels as does Ca^{2+}. Taken together, these results raise the possibility that adenosine activates a calcium channel. To the best of our knowledge, this is a novel intracellular signaling pathway for adenosine receptors. Adenosine A_1 receptors are known to be coupled to a variety of second messenger systems, including inhibition of adenylate cyclase, activation of potassium channels, and modulation of inositol phosphates (17), whereas adenosine A_2 receptors are thought to act mostly through activation of adenylate cyclase (17). In most systems, A_2 receptor-mediated increases in cAMP are associated with a negative modulation of intracellular Ca^{2+} levels, as seen in platelets (6) and in vascular smooth muscle cells (18), resulting in inhibition of aggregation and vasodilatation.

Of interest, it has recently been reported that A_{2b} receptors potentiate a P-type calcium current in hippocampal neurons (19). Adenosine enhances the release of glutamate in the hippocampus (20), whereas it depresses neural function and inhibits the release of virtually all neurotransmitters in most other brain regions and in efferent nerve fibers. Paradoxical excitatory actions are also found in the nucleus tractus solitarii, where adenosine also enhances the release of glutamate (21), and in afferent sensory and chemosensitive fibers (22). It remains to be determined whether these apparent excitatory actions of adenosine are uniformly characterized by increased calcium influx due to activation of A_{2b} receptors via coupling to G_s proteins, as we have found in HEL cells.

Based on our results, we cannot determine the precise mechanism, downstream from G_s protein activation, by which adenosine increases Ca^{2+} influx. To adequately explain our findings, such a mechanism would require the activation of α_s subunits, given the sensitivity to cholera toxin, and an initial increase in intracellular Ca^{2+} levels, given that this phenomenon was not observed in the absence of thrombin, PGE_1, a calcium ionophore, or thapsigargin. From our studies we cannot determine the mechanism by which this initial rise in intracellular Ca^{2+} is required for the coupling between the adenosine receptor-activated G_s protein and the putative Ca^{2+} channel. Whether this is the result of a direct action of Ca^{2+} or occurs indirectly, e.g., through activation of a Ca^{2+}-sensitive protein kinase, remains to be determined. Likewise, we could speculate that the increase in Ca^{2+} influx is mediated directly through the α subunit of G_s, as suggested from β-adrenoreceptor-mediated effects on voltage-gated Ca^{2+} current in the myocardium (23). Alternatively, the effects of adenosine on Ca^{2+} influx may be mediated by the $\beta\gamma$ subunits through a mechanism that requires α_s subunit activation. In other systems, for example, the activation of adenylate cyclase by the $\beta\gamma$ complex requires costimulation of α_s-GTP (24). Of interest, $\beta\gamma$ subunits mediate the stimulation of muscarinic receptor-gated potassium channels in the heart (25). These potassium channels are also activated by adenosine A_1 receptors in a GTP-dependent manner, suggesting that $\beta\gamma$ subunits underlie these effects.

The effect of adenosine on intracellular Ca^{2+} is lost if HEL cells are differentiated with phorbol esters. We have previously shown that adenosine-induced cAMP accumulation is selectively lost in differentiated cells, whereas the responses to iloprost and forskolin are maintained (3, 5). It is possible that differentiation alters the expression of adenosine receptors or their coupling to G_s proteins. It has been shown, for example, that differentiation of HEL cells alters the expression of membrane receptors (3) and cell surface markers (1).

In addition to the modulatory effects of adenosine, we found that PGE_1, but not prostacyclin, increased intracellular Ca^{2+} levels through a pertussis toxin-sensitive mechanism, in agreement with recent reports (26, 27). Furthermore, we found that, whereas iloprost was more potent than PGE_1 in increasing cAMP, the reverse was true for the rise in intracellular Ca^{2+} (Fig. 1). The differences in potencies and in coupling to G proteins suggest that HEL cells possess distinct receptors for PGE_1 and for prostacyclin. In contrast, it has been suggested that PGE_1 and prostacyclin act through the same receptor in human platelets (28). Therefore, membrane receptors for prostaglandins and adenosine, and the coupling of these receptors to intracellular signaling pathways, are markedly different in HEL cells and human platelets. Whereas HEL cells are useful for studying intracellular signaling pathways, these findings raise doubts about their suitability as models for studying human platelets.

In summary, we report a novel intracellular signaling pathway for adenosine A_2 receptors. Activation of A_{2b} receptors in HEL cells results in parallel but independent increases in cAMP and intracellular Ca^{2+} levels via cholera toxin-sensitive mechanisms. The rise in intracellular Ca^{2+} levels produced by adenosine requires a synergistic action with other agents that increase basal intracellular Ca^{2+} levels, such as thrombin, PGE_1, calcium ionophore, or thapsigargin. The precise mechanism of this effect remains to be determined, but influx of extracellular calcium appears to contribute significantly to the rise in intracellular Ca^{2+} produced by adenosine. It is not yet known whether this intracellular signaling pathway is universally shared by A_{2b} receptors found in other cells and whether this mechanism explains the increasingly well recognized excitatory actions of adenosine.

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