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The role of adenosine A_1 receptors in the ATP-evoked Ca^{2+} response in rat thyroid FRTL-5 cells

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Abstract

The effect of adenosine A_1 receptor activation on the ATP-induced increase in intracellular free Ca^{2+} was studied in control and protein kinase C down-regulated Fisher rat thyroid (FRTL-5) cells. Long-term phorbol ester treatment, which leads to protein kinase C down-regulation, enhanced the ATP-evoked extracellular Ca^{2+} influx. The increased Ca^{2+} influx was antagonized by the adenosine A_1 receptor antagonist 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX). [^{3}H]DPCPX binding studies revealed that phorbol ester-treatment increased the number of adenosine A_1 receptors. The adenosine A_1 receptor-mediated inhibition of the cyclic AMP formation was not affected by the increased receptor number. We conclude that the enhanced ATP-evoked Ca^{2+} influx in protein kinase C down-regulated cells is mediated by adenosine formed by hydrolysis of ATP, and that this adenosine interacts with the increased number of A_1 receptors. The mechanism by which adenosine enhances Ca^{2+} entry is not known. Thus, the larger number of adenosine A_1 receptors broadens the spectrum of adenosine A_1 receptor affected signaling systems in FRTL-5 cells.

Keywords: Adenosine A_1 receptor; ATP; FRTL-5 thyroid cell; Protein kinase C; Signal transduction

1. Introduction

Extracellular purines, e.g., ATP and adenosine, act on different types of membrane bound receptors. ATP and other nucleotides mediate their action via G protein-coupled P2Y receptors or ion channels called P2X receptors (Ralevic and Burnstock, 1998). P2Y receptors show partial sensitivity to pertussis toxin and are able to couple to two distinct families of G proteins; either pertussis toxin-insensitive $G_{q/11}$ proteins, which activate phospholipase C or pertussis toxin-sensitive $G_{i/o}$ proteins connected to inhibition of adenylyl cyclase (Ralevic and Burnstock, 1998). Adenosine activates G protein-coupled A_1, A_{2A}, A_{2B} and A_3 receptor subtypes (Olah and Stiles, 1995). Adenosine A_1 receptors couple to pertussis toxin-sensitive $G_{i/o}$ proteins (Munshi et al., 1991), which leads to stimulation of several effector systems including inhibition of adenylyl cyclase (Van Calker et al., 1978) and activation of phospholipase C (Gerwins and Fredholm, 1992a,b).

The ATP-mediated Ca^{2+} response in FRTL-5 cells has been extensively studied (Törnquist, 1991a,b; Törnquist, 1992, 1993; Törnquist and Ekokoski, 1995). It is composed of a rapid release of Ca^{2+} from intracellular stores, followed immediately by an increase in the influx of extracellular Ca^{2+} (Törnquist, 1991b; Törnquist, 1992). Acute activation of protein kinase C by the phorbol ester phorbol-12-myristate-13-acetate (PMA) decreases the ATP-evoked increase in intracellular free Ca^{2+} [Ca^{2+}]$_i$ in FRTL-5 cells (Törnquist, 1993). In protein kinase C down-regulated FRTL-5 cells, where protein kinase C activity is reduced by long-term activation of protein kinase C by PMA (Akiguchi et al., 1993), the P2 receptor-mediated increase in [Ca^{2+}]$_i$ is enhanced (Törnquist, 1993;

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Shimegi et al., 1994). Especially the ATP-evoked, pertussis toxin-sensitive, influx of extracellular Ca^{2+} is increased by protein kinase C down-regulation (Törnquist and Ekokoski, 1995), indicating that down-regulation of protein kinase C modulates the $G_{i/o}$-mediated signaling. In addition, the fact that protein kinase C down-regulation had no effect on the pertussis toxin-insensitive GTP-evoked response (Törnquist and Ekokoski, 1995) supports this hypothesis. The pertussis toxin-sensitive part of the ATP-mediated response was found to be antagonized by adenosine A_1 receptor antagonists (Okajima et al., 1989a), suggesting an activation of adenosine A_1 receptors. In FRTL-5 cells activation of adenosine A_1 receptors per se has no effect on $[Ca^{2+}]_i$ (Okajima et al., 1989a, 1997; Sho et al., 1991). Nevertheless, adenosine A_1 receptor agonists have been shown to enhance Ca^{2+} responses evoked by GTP (Okajima et al., 1989a; Shimegi et al., 1994), TSH (Sho et al., 1991), sphingosine 1-phosphate (Okajima et al., 1997) and noradrenaline (Okajima et al., 1989b). In other cell types, like DDT_1 MF-2 smooth muscle cells, activation of A_1 receptors alone increase $[Ca^{2+}]_i$ and this effect acts synergistically with the ATP-evoked mobilization of intracellular Ca^{2+} (Gerwins and Fredholm, 1992a). In DDT_1 MF-2 cells, part of the ATP-mediated response was attenuated with adenosine deaminase treatment, suggesting a rapid degradation of ATP to ADP, AMP and adenosine (Gerwins and Fredholm, 1992a). The degradation of ATP to its metabolites only partially explains the $G_{i/o}$-mediated effects of ATP in FRTL-5 cells (Sato et al., 1992), and ligand binding experiments with slowly-hydrolyzable ATP analogues has been used to prove that ATP occupies both adenosine A_1 receptor antagonist sensitive and insensitive binding sites (Okajima et al., 1989a).

It appears that pertussis toxin-sensitive, $G_{i/o}$-oupled adenosine A_1 receptor activation is closely connected to ATP-mediated responses in FRTL-5 cells. The pertussis toxin-sensitive, $G_{i/o}$-coupled ATP-mediated increase in $[Ca^{2+}]_i$ is enhanced in protein kinase C down-regulated FRTL-5 cells (Törnquist and Ekokoski, 1995). Therefore, we decided to investigate the function of adenosine A_1 receptors on the enhanced ATP-mediated increase in $[Ca^{2+}]_i$ after protein kinase C down-regulation to further clarify the role of adenosine A_1 receptors in ATP-mediated effects.

2. Materials and methods

2.1. Materials

Culture medium and hormones needed for the cell culture were purchased from Sigma (St Louis, MO). Serum was from Biological Industries (Beth Haemek, Israel). Culture dishes were from Falcon Plastics (Oxnard, CA). Thyrotropin (TSH) was a generous gift from the National Hormone and Pituitary Program and Dr. A.F. Parlow (Bethesda, MD). Tritium labeled 1,3-dipropyl-, 8-cyclopentyl xanthine ([^{3}H]DPCPX, 120 Ci/mmol) and adenosine 3′, 5′-cyclic phosphate ([^{3}H]cAMP, 25 Ci/mmol) were from New England Nuclear (Boston, MA). Adenosine deaminase was from Boehringer Mannheim (Germany). 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) was purchased from Calbiochem (La Jolla, CA). Phorbol-12-myristate-13-acetate (PMA) and N^6-cyclohexyladenosine (CHA) were purchased from Sigma. Fura 2 acetoxylmethyl ester (Fura 2-AM) was purchased from Molecular Probes (Eugene, OR), and thapsigargin from LC Services (Woburn, MA). MultiScreen-FB plates were from Millipore (Espoo, Finland). OptiPhase HiSafe III and SuperMix scintillation fluids were from Wallac (Turku, Finland).

2.2. Cell culture

Rat thyroid FRTL-5 cell line was a generous gift from Dr. Egil Haug (Aakers Hospital, Oslo, Norway) and was initially obtained from Interthyr Research Foundation. The cells were cultured in Coon's modified Ham's F-12 medium supplemented with 5% newborn calf serum and six hormones (TSH 0.3 mU/ml, insulin 1 μg/ml, transferrin 5 μg/ml, somatostatin 10 ng/ml, hydrocortisone 10 nM, the tripeptide Gly-L-His-L-Lys 10 ng/ml) (Ambesi-Impiombato et al., 1980). The cells were grown on 10-cm cell culture dishes to confluence in the presence of TSH. Three days prior to the experiments TSH was withdrawn from the culture medium. Protein kinase C was down-regulated by the treatment with 1 μM PMA for 24 h (Akiguchi et al., 1993).

2.3. Radioligand binding assay

Adenosine A_1 receptors were quantified using [^{3}H]DPCPX binding on intact FRTL-5 cells in suspension. Cells were harvested with a 0.2 % trypsin solution and washed three times with phosphate buffered saline (PBS). The cells were resuspended in serum-and hormone-free Coon's F-12 medium buffered with 20 mM HEPES (pH 7.4) and supplemented with 0.1 % bovine serum albumin, at a density of 3×10^6 cells/ml. In saturation binding studies cells (0.1 ml, 0.3×10^6 cells) were added to test tubes containing [^{3}H]DPCPX (0.03–6 nM) with or without cold ligand. Nonspecific binding was defined as that occurring in the presence of 40 μM CHA. In [^{3}H]DPCPX displacement studies, the cells were incubated with 1 nM [^{3}H]DPCPX and with various concentrations of competing ligand. The Cheng–Prusoff correction was used in the K_i calculations. In both assays, the incubation time was 2 h at room temperature. Assays were terminated by rapid filtration through glass fiber filters (Whatman GF/C) on a 12-well harvester system, and the filters were rapidly washed 3 times with 3 ml of ice-cold PBS. The filters were then dried and transferred to scintillation vials.

2.4. Measurement of $[Ca^{2+}]_i$

The method was as described recently (Törnquist et al., 1999), using the fluorescent probe Fura 2-AM. The $[Ca^{2+}]_i$ values were calculated as described earlier (Grynkiewicz et al., 1985) using a computer program developed for the Hitachi F2000 fluorometer. The K_D value for Fura 2 was 224 nM. In the experiments made in Ca^{2+}-free buffer, $CaCl_2$ was omitted from incubation buffer and 100 μM EGTA was added to the cuvette prior to measurement. The Ca^{2+} entry was studied after addition of 1 mM $CaCl_2$.

2.5. Measurement of cyclicAMP

Cells grown without TSH for 3 days and treated with or without PMA (1 μM, 24 h) were harvested with a 0.2 % trypsin solution and washed three times with HEPES-buffered saline solution (HBSS, in mM concentrations: NaCl, 118; KCl, 4.6; $CaCl_2$, 0.4; HEPES, 10; glucose, 10; pH 7.2). The cells were resuspended in HBSS containing 1 U/ml adenosine deaminase and 0.1 % bovine serum albumin. Aliquots (0.35 ml, 0.5×10^6 cells) were added to test tubes and incubated 20 min at 37°C. Forskolin (final concentration of 1 μM) with or without CHA (final concentration of 0.01-100 nM) was added to the test tubes together with the phosphodieterase inhibitor Ro 20-1724 (final concentration of 100 μM). The cells were incubated with test reagents for 20 min at 37°C. The incubation was stopped with 50 μl perchloric acid to a final concentration of 0.1 M. The samples were neutralized by KOH and the cAMP content in the supernatants was determined by a protein binding method (Nordstedt and Fredholm, 1990). In brief, the samples and standards (0–160 nM cAMP) were incubated with protein extract (40 μg) from bovine adrenal cortex containing cAMP binding protein and with [^{3}H]cAMP (10, 000 cpm/tube) for 2.5 h at 4°C. The bound cAMP was separated from free cAMP by rapid filtration over MAFB Millipore MultiScreen filtration plates and the wells were washed 3 times with 250 μl HBSS. The plates were dried and 50 μl SuperMix scintillation fluid was added to the wells. The plates were counted using a MicroBeta scintillation counter (Wallac, Finland).

2.6. Statistics

Data are given as the mean ± S.E.M. from at least three experiments. Statistical comparisons were made from the data using Student's *t*-test, with the probability level of $P < 0.05$ being considered significant. The log-values were used to compare EC_{50}-values. Analysis of variance was used when three or more means were tested. Data from receptor binding studies were analyzed by nonlinear regression for receptor binding, using GraphPad Prism ligand binding software for Macintosh computer (GraphPad Software, San Diego, CA).

3. Results

3.1. Protein kinase C down-regulation enhances ATP-evoked Ca^{2+} responses

Down-regulation of protein kinase C enhanced the 0.3 μM ATP-evoked increase in intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$ by 78 %. The ATP-evoked increase in $[Ca^{2+}]_i$ was 342 ± 32 nM in control cells and 610 ± 56 nM in PMA-treated cells. It has been shown that the increased effect of ATP in protein kinase C down-regulated cells is largely dependent on influx of extracellular Ca^{2+} (Törnquist, 1993). This was confirmed in the present study. The ATP-evoked response was significantly lower in Ca^{2+}-free buffer (225 ± 24 nM in control cells and 199 ± 43 nM in PMA-treated cells), and no difference in ATP-evoked response was detected between control and protein kinase C down-regulated cells. Representative Ca^{2+} traces are shown in Fig. 1.

3.2. Effect of DPCPX on ATP-mediated increases in $[Ca^{2+}]_i$

The adenosine A_1 receptor antagonist DPCPX (10^{-10}–10^{-7} M) dose-dependently inhibited the Ca^{2+} response evoked by 0.3 μM ATP in control and in protein kinase C down-regulated cells (Fig. 2A). Protein kinase C down-regulated cells were more sensitive to the inhibitory effect of DPCPX compared with control cells. The inhibition of 0.3 μM ATP-evoked response obtained with 100 nM DPCPX, was 27 ± 4 % in control cells and 49 ± 5 % in

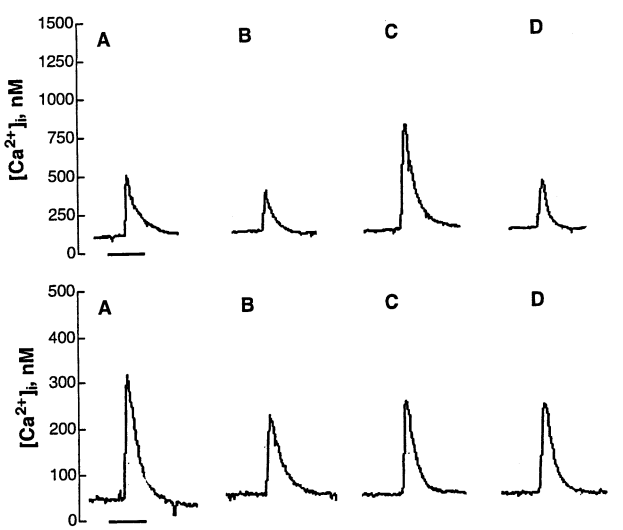

Fig. 1. Representative recordings of ATP-evoked increase in $[Ca^{2+}]_i$. The cells in the upper row were stimulated with 0.3 μM ATP in Ca^{2+}-containing buffer, and the cells in the lower row were stimulated with 0.3 μM ATP in Ca^{2+}-free buffer. Traces A and B are from control cells and C and D from protein kinase C down-regulated cells. In traces B and D 100 nM DPCPX was added to the cells 2 min before ATP stimulation. The horizontal bar denotes 1 min. Note the difference in the ordinates.

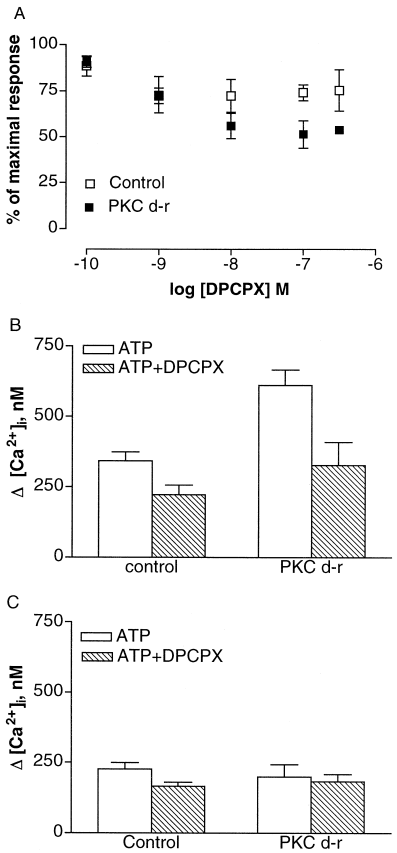

Fig. 2. Summary of the effects of DPCPX on ATP-evoked $[Ca^{2+}]_i$ increase. In panel A., the dose-dependent inhibition of the 0.3 μM ATP-evoked increase in $[Ca^{2+}]_i$ by DPCPX in control and protein kinase C down-regulated cells is shown as the percentage of ATP-evoked response. In panel B and C, the effect of 100 nM DPCPX in control cells and protein kinase C down-regulated cells is shown in Ca^{2+}-containing buffer (B), and in Ca^{2+}-free buffer (C). DPCPX inhibited the ATP-evoked response in Ca^{2+}-containing buffer but not in Ca^{2+}-free buffer ($P < 0.05$). Results shown are means ± S.E.M. of four to eight separate experiments.

PMA-treated cells (Fig. 2B). DPCPX (100 nM), added 2 min before the ATP stimulation, had no effect on basal $[Ca^{2+}]_i$ (data not shown). In Ca^{2+}-free buffer, DPCPX was without an effect on the ATP-induced increase in $[Ca^{2+}]_i$ (Fig. 2C). Representative Ca^{2+} traces are shown in Fig 1. The treatment with 100 nM DPCPX prior to stimulation with different concentrations of ATP (0.1–100 μM) induced a rightward shift in the dose-response curve for ATP without affecting the maximal response (Fig. 3). The EC_{50} values for control and DPCPX-treated cells were 0.24 μM (0.16–0.36 μM 95 % confidence interval) and 0.61 μM (0.40–0.94 μM 95 % confidence interval), respectively.

To exclude an effect of DPCPX on capacitative Ca^{2+} entry (Putney and Bird, 1993), the effect of 100 nM DPCPX on thapsigargin-evoked Ca^{2+} entry was studied in protein kinase C down-regulated cells. In cells treated with vehicle or DPCPX, the thapsigargin-evoked Ca^{2+} release in Ca^{2+}-free buffer was of similar magnitude (84 ± 9 nM in vehicle-treated cells and 68 ± 10 nM in DPCPX-treated cells). Furthermore, readdition of 1 mM Ca^{2+} to these

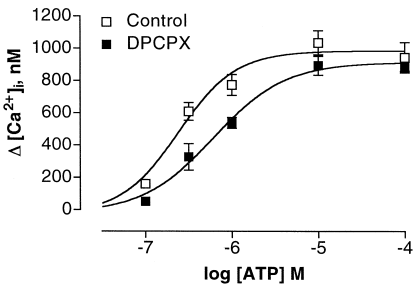

Fig. 3. The effect of 100 nM DPCPX on EC_{50}-values for ATP-evoked increase in $[Ca^{2+}]_i$ in protein kinase C down-regulated cells. The cells were treated with or without 100 nM DPCPX 2 min before ATP stimulation. DPCPX shifted the EC_{50} value for ATP from 0.2 μM (0.16–0.36 μM 95 % confidence interval) to 0.6 μM (0.40–0.94 μM 95 % confidence interval). The results shown are the mean ± S.E.M. from four separate experiments.

cells increased $[Ca^{2+}]_i$ by 824 ± 93 nM in control cells and 842 ± 181 nM in DPCPX-treated cells.

3.3. Effect of adenosine deaminase on ATP-mediated increases in $[Ca^{2+}]_i$

Treatment with adenosine deaminase (1 mU/ml, at least 30 min preincubation and throughout the experiments) had no effect on 0.3 μM ATP-mediated increase in $[Ca^{2+}]_i$ in protein kinase C down-regulated cells (610 ± 56 nM in control and 569 ± 35 nM in the presence of adenosine deaminase, Fig. 4). However, treatment with adenosine deaminase significantly reduced the effect of 0.3 μM ATPγS on $[Ca^{2+}]_i$ (458 ± 58 nM in control cells and 193 ± 32 nM in the presence of adenosine deaminase).

3.4. Enhanced [^{3}H] DPCPX binding in protein kinase C down-regulated cells

Treatment with 1 μM PMA time-dependently increased the adenosine A_1 receptor number (Fig. 5A). The increase in maximal binding (B_{max}) was 107 % in cells treated wit 1 μM PMA for 24 h (B_{max} 58 ± 5 fmol/10^6 cells)

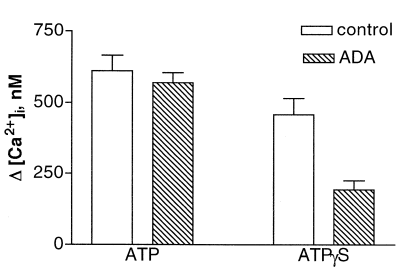

Fig. 4. The effect of adenosine deaminase on the ATP-and ATPγS-evoked increase in $[Ca^{2+}]_i$ in protein kinase C down-regulated cells. The cells were treated with or without 1 U/ml adenosine deaminase (ADA) at least 30 min before ATP stimulation. Adenosine deaminase inhibited the 0.3 μM ATPγS-evoked increase in $[Ca^{2+}]_i$ ($P < 0.05$) but was without any effect on the 0.3 μM ATP-evoked response. Results shown are means ± S.E.M. from four to six separate experiments.

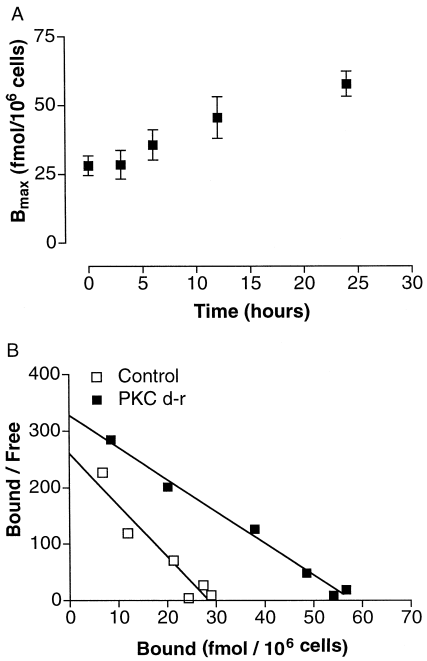

Fig. 5. The effect of PMA on [^{3}H]DPCPX binding. In panel A, B_{max} values (fmol/10^6 cells) were determined from [^{3}H]DPCPX saturation experiments performed on cells treated with 1 μM PMA for the indicated periods of time. Results shown are means ± S.E.M. from three separate experiments. In panel B, Scathcard plots from the control and protein kinase C down-regulated (1 μM PMA, 24 h) cells are shown. B_{max} values for control and protein kinase C down-regulated cells were 28 ± 4 fmol/10^6 cells and 58 ± 5 fmol/10^6 cells, respectively. No statistically demonstrable change in K_D values was detected (0.11 ± 0.02 nM in control cells and 0.17 ± 0.01 nM in protein kinase C down-regulated cells). Scatchard plot data is collected from three separate [^{3}H]DPCPX saturation experiments.

compared to control cells (28 ± 4 fmol/10^6 cells). At least 12 h were needed to obtain the increased receptor number. Protein kinase C down-regulation had no statistically demonstrable effect on adenosine A_1 receptor affinity (Fig. 5B). The K_D values for control and PMA-treated cells were 0.11 ± 0.02 and 0.17 ± 0.01 nM, respectively. The binding of [^{3}H]DPCX (0.03–30 nM) represented one binding site model when one and two binding site models were compared with GraphPad Prism ligand binding software (data not shown).

3.5. Effect of ATPγS on [^{3}H]DPCPX binding

The binding of the slowly hydrolyzable ATP analog ATPγS to the DPCPX-sensitive adenosine A_1 receptor was characterized in protein kinase C down-regulated cells by displacement analysis using 1 nM [^{3}H]DPCPX (Fig. 6). ATPγS displaced bound [^{3}H]DPCPX with calculated K_i value of 2.5 μM (1.9–3.2 μM 95 % confidence interval). The ATPγS-mediated displacement was compared with the adenosine A_1 receptor agonist CHA-mediated displacement of [^{3}H]DPCPX, which gave a K_i value of 47 nM (33–66 nM 95% confidence interval). When the analysis

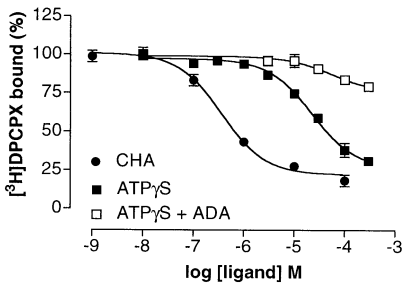

Fig. 6. Displacement of 1 nM [^{3}H]DPCPX by ATPγS and CHA. The protein kinase C down-regulated cells were incubated with 1 nM [^{3}H]DPCPX and indicated concentrations of ligands for 2 h at room temperature. ATPγS displaced bound [^{3}H]DPCPX with calculated K_i value of 2.5 μM (1.9–3.2 μM 95 % confidence interval) in the absence of adenosine deaminase. ATPγS-mediated displacement of bound [^{3}H]DPCPX was abolished in the presence of adenosine deaminase (ADA, 1 U/ml). CHA-mediated displacement of [^{3}H]DPCPX gave a K_i-value of (33–66 nM 95% confidence interval). The values are expressed as percentages of [^{3}H]DPCPX binding obtained in the absence of competing ligands. Results shown are means ± S.E.M. from six separate experiments.

was done in the presence of adenosine deaminase (1 U/ml) the effect of ATPγS was abolished. In intact cells binding occurs to a low affinity site (Gerwins et al., 1990) and the K_i values were similar in control cells compared with protein kinase C down-regulated cells (data not shown).

3.6. Adenylyl cyclase inhibition via adenosine A_1 receptor was not affected by protein kinase C down-regulation

The protein kinase C down-regulated and control cells were stimulated with 1 μM forskolin to activate adenylyl cyclase. CHA (10^{-11}–10^{-6} M) was used to inhibit the formation of cAMP. The increase in receptor number in

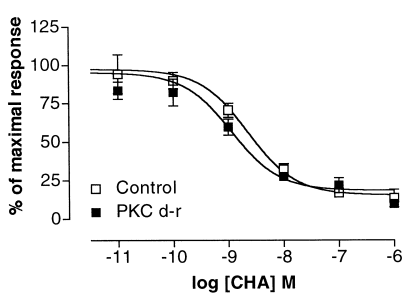

Fig. 7. CHA-mediated inhibition of cAMP formation in control and protein kinase C down-regulated cells. The figure shows the ability of CHA to inhibit the formation of cAMP induced by 1 μM forskolin. Results are expressed as percentage of forskolin-induced cAMP accumulation. The basal level of cAMP (6.4 ± 0.8 nM vs. 6.3 ± 0.8 nM) and the forskolin-induced response (40 ± 8 nM vs. 31 ± 6 nM) were similar in control and protein kinase C down-regulated cells. IC_{50} values for CHA were similar in control and protein kinase C down-regulated cells: 2 nM (1.2–3.4 nM 95% confidence interval) in control cells, and 1 nM (0.6–1.6 nM 95% confidence interval) in protein kinase C down-regulated cells. Results shown are means ± S.E.M. from five separate experiments.

protein kinase C down-regulated cells had no effect on adenosine A_1 receptor-mediated inhibition of adenylyl cyclase (Fig. 7). The IC_{50} values for CHA were 2 nM (1.2–3.4 nM 95 % confidence interval) in control cells and 1 nM (0.6–1.6 nM 95 5 confidence interval) in protein kinase C down-regulated cells. The basal level of cAMP was similar in control (6.4 ± 0.8 nM) and protein kinase C down-regulated cells (6.3 ± 0.8 nM). Furthermore, the forskolin-evoked response in cAMP was not different in control cells, compared with that in protein kinase C down-regulated cells: 40 ± 8 and 31 ± 6 nM, respectively.

4. Discussion

In the present report, we show that the ATP-evoked increase in $[Ca^{2+}]_i$ was enhanced after long-term phorbol ester treatment (i.e. down-regulation of protein kinase C activity), a result that we also have observed previously (Törnquist, 1993). In the present study we have used an ATP concentration close to the EC_{50} for ATP, which allows a more sensitive modulation of the response than supramaximal concentrations of ATP (100 μM) used in previous studies (Törnquist, 1993; Törnquist and Ekokoski, 1995).

Activation of adenosine A_1 receptors has been shown to increase the P2Y receptor –mediated IP_3 formation and intracellular Ca^{2+} increase in FRTL-5 cells (Okajima et al., 1989a). To study the role of adenosine A_1 receptor activation in the increased response for ATP after protein kinase C down-regulation we used the adenosine A_1 receptor specific antagonist DPCPX (Lohse et al., 1987). The inhibitory effect of DPCPX on the ATP-mediated increase in $[Ca^{2+}]_i$ was enhanced in protein kinase C down-regulated cells, suggesting that PMA-treatment increased the participation of the adenosine A_1 receptors in the ATP-mediated response. DPCPX shifted the dose-response curve for ATP to the right without affecting the maximal response, indicating that the action of adenosine A_1 receptors was more important with submaximal concentrations of ATP.

The [^{3}H]DPCPX saturation binding revealed an increased adenosine A_1 receptor number in protein kinase C down-regulated cells. The conclusion from previous studies with ATP (Törnquist, 1993; Törnquist and Ekokoski, 1995) has been that activation of protein kinase C possible modulates a second messenger-operated Ca^{2+} channel. However, the results from the present study suggest that the increased adenosine A_1 receptor number mediates the enhanced ATP-evoked increase in $[Ca^{2+}]_i$ in protein kinase C-down-regulated cells. This conclusion is supported by the finding that the protein kinase C inhibitor staurosporine did not enhance the ATP-induced increase in $[Ca^{2+}]_i$ in the previous study (Törnquist, 1993).

In DDT_1 MF-2 cells, the ATP-evoked IP_3 formation and increase in $[Ca^{2+}]_i$ is enhanced by the breakdown of ATP into adenosine and a concomitant adenosine-evoked activation of adenosine A_1 receptors (Gerwins and Fredholm, 1992a). In the present study, treatment with adenosine deaminase had no statistically significant effect on the ATP-increased $[Ca^{2+}]_i$, but adenosine deaminase did reduce the effect of the more slowly hydrolyzable ATP analog ATPγS. As ATPγS has been previously suggested to bind to adenosine A_1 receptors in FRTL-5 cells (Okajima et al., 1989a; Sato et al., 1992), we investigated whether ATPγS displaced [^{3}H]DPCPX from the adenosine A_1 receptors in the presence or absence of adenosine deaminase. ATPγS displaced all of the specifically bound [^{3}H]DPCPX with a low micromolar K_i value in the absence of adenosine deaminase. However, the effect was abolished in the presence of adenosine deaminase. The result suggests that adenosine deaminase, present in the measurement of $[Ca^{2+}]_i$, is not effectively degrading adenosine generated by the fast hydrolysis of ATP by membrane bound ectonucleotidases (Gordon, 1986), but effectively degrades adenosine generated by the slow hydrolysis of ATPγS.

In our study, DPCPX decreased the ATP-evoked influx of extracellular Ca^{2+}. The inhibitory effect of DPCPX was not shown in control or PMA-treated cells stimulated by ATP in Ca^{2+}-free buffer. This indicates that adenosine A_1 receptors are not only connected to phospholipase C activation but also to the Ca^{2+} conductance of the cell membrane. We tested whether the effect of DPCPX on Ca^{2+} influx was receptor specific or related to other membrane functions, e.g. capacitative Ca^{2+} entry. DPCPX did not affect capacitative Ca^{2+} entry, in which Ca^{2+} influx occurs through non-receptor operated Ca^{2+} channels. The low concentration of ATP (0.3 μM) used in this study induces a very rapid and transient increase in $[Ca^{2+}]_i$ without an elevated plateau phase seen with the higher ATP concentrations. If activation of adenosine A_1 receptor affects the influx of extracellular Ca^{2+} as suggested, the effect should also be seen in the plateau formed by influx of extracellular Ca^{2+}. This type of adenosine A_1 receptor-mediated potentiation of $[Ca^{2+}]_i$ has been shown in FRTL-5 cells treated with GTP (Okajima et al., 1989a), TSH (Sho et al., 1991), and sphingosine 1-phosphate (Okajima et al., 1997). In these studies, the adenosine A_1 receptor-mediated potentiation of $[Ca^{2+}]_i$ was related to synergistic actions on phospholipase C, and the mechanism of the enhanced influx of extracellular Ca^{2+} was not studied.

Adenosine A_1 receptor activation inhibits adenylyl cyclase via activation of the α_i subunit of the trimeric $G_{i/o}$ protein, whereas the $\beta\gamma-$ subunit is responsible for the augmentation of phospholipase C-mediated effects (Selbie et al., 1997; Tomura et al., 1997). High level of cAMP has been shown to inhibit ATP-mediated Ca^{2+} responses in FRTL-5 cells (Törnquist and Ahlström, 1993; Laglia et al., 1996). In the present study, cells grown without TSH were used to reduce the cAMP-mediated regulation of the Ca^{2+} signaling. The basal cAMP level and the increase in cAMP induced by forskolin-treatment were similar in control

cells and in protein kinase C down-regulated cells. When adenosine A_1 receptor-mediated inhibition of forskolin-induced cAMP formation was studied, no change was found in the efficiency of adenosine A_1 receptors to inhibit adenylyl cyclase after protein kinase C down-regulation. According to Gudermann et al., 1996, the inhibition of adenylyl cyclase via $G\alpha_i$ proteins occurs at low agonist concentrations and is independent of receptor density. However, phospholipase C activation by $\beta\gamma$-subunit requires considerably higher concentrations of ligand and is directly correlated to A_1 receptor abundance (Biber et al., 1997). This supports the finding that the effect of adenosine A_1 receptors on Ca^{2+} signaling is more sensitive to the receptor up-regulation than adenylyl cyclase inhibition, but does not explain the mechanism for adenosine A_1 receptor-mediated Ca^{2+} influx.

In summary, we have shown that the enhanced ATP-evoked increase in $[Ca^{2+}]_i$ in protein kinase C down-regulated cells is mediated via increased adenosine A_1 receptors, probably activated by adenosine generated by the degradation of ATP. In addition to the previously shown potentiation of phospholipase C-mediated IP_3 formation, activation of adenosine A_1 receptors modulated the ATP-evoked influx of extracellular Ca^{2+}. The adenosine A_1 receptor regulated influx of extracellular Ca^{2+} was shown to be more sensitive to the increased receptor number than the inhibition of adenylyl cyclase. These results indicate more complex ''cross-talk'' between ATP and adenosine A_1 receptor-mediated signals than suggested previously.

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