A1 and A2 Adenosine Receptor Activation Inversely Modulates Potassium Currents and Membrane Potential in DDT1 MF-2 Smooth Muscle Cells

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ABSTRACT—Adenosine receptors are widely distributed in mammalian tissues and have been possibly involved through transmembrane potential changes in cell function regulation. The effect of A1 and A2A adenosine receptor ligands on transmembrane potential measured with flow cytometry and potassium conductance measured by the patch-clamp technique was investigated in DDT1 MF-2 smooth muscle cells. The A1 adenosine-receptor agonist CPA (50 nM) and the A2A adenosine-receptor agonist CGS 21680 (50 nM) elicited a rapid and maintained increase and decrease in the potassium conductance, respectively, and a concomitant hyperpolarization and depolarization of the membrane, respectively. These effects were eliminated by subtype-selective adenosine receptor antagonists (DPCPX, CSC, ZM 241385, all 1/G6d). The ligand induced membrane potential changes were reversible. Based on these detected membrane potential changes along with the published voltage dependence of the adenylyl cyclase, the regulation of cAMP production by A1- and A2A-receptor activation is suggested to be mediated through the induced early hyperpolarization and depolarization. The interaction between the effects of these receptor subtypes allows for a complex regulation mechanism.

Keywords: A1 adenosine receptor, A2 adenosine receptor, Potassium channel, Membrane potential, DDT1 MF-2 cell

Adenosine is an endogenous modulator of cellular functions in the central nervous system as well as in the periphery. Adenosine receptors are widely distributed in mammalian tissues and have been divided into four subtypes called A1, A2A, A2B and A3 (1). These are seven-transmembrane domain proteins coupled to regulatory G-proteins that inhibit (A1 and A3 receptors) or stimulate (A2A and A2B receptors) adenosine 3′:5′-cyclic monophosphate (cAMP) formation. The adenosine A3 receptor has high affinity (EC50 = 0.1 – 1 μM) and the A2B receptor low affinity (EC50 = 5 – 10 μM) for adenosine (2 – 5). Although the most widely recognised signalling pathway of adenosine receptors is inhibition or activation of adenylyl cyclase activity, many of the effects of adenosine are coupled to conductance changes, particularly the modification of K+ and Ca2+ channels involving, of course, also an alteration in membrane potential (6 – 9). Membrane potential is known to have a crucial role in signal transduction. However, the possible involvement of transmembrane potential change in the response of cells to adenosine-receptor activation has not been studied in detail so far. The aim of this study was to investigate the effect of the interaction of A1 and A2A adenosine receptors and their ligands on the potassium conductance of the membrane and membrane potential of the cells. DDT1 MF-2 smooth muscle cells derived from hamster vas deferens (10) express both A1 and A2A receptor subtypes (11, 12) and voltage-dependent potassium channels characterized by Molleman et al. (13); thus they offer an appropriate experimental system for these investigations.

MATERIALS AND METHODS

Reagents
All of the applied chemicals were of analytical or
spectroscopic grade. Bis(1,3-dibutylbarbituric acid(5)) trimethine oxonol (diBA-Cr(3)) (oxonol) was obtained from Molecular Probes (Eugene, OR, USA). Adenosine deaminase was from Boehringer-Mannheim (Indianapolis, IN, USA). The adenosine receptor ligand 4-(2-[7-aminocarbonyl]-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino)ethyl)phenol (ZM 241385) was obtained from Tocris (Bristol, UK). (2-p-ethyl)phenol (ZM 241385) was obtained from RBI (Bristol, UK). (2-p-ethyl)phenol (ZM 241385) was obtained from RBI (Bristol, UK). (2-p-ethyl)phenol (ZM 241385) was obtained from RBI (Bristol, UK). 2-(2-furyl)[1,2,4]triazolo[2,3-]

IN, USA). The adenosine receptor ligand 4-(2-[7-amino-

decarboxamidoadenosine (CGS 21680) and 8-cyclo-
pentyl 1,3-dipropylxanthine (DPCPX) were from RBI (Natick, MA, USA). 8-(3-Chlorostyryl)caffeine (CSC) was prepared as previously described (14). Stock solutions of the dye diBA-Cr(3) and adenosine receptor ligands were prepared in DMSO and stored at −20°C in the dark. Fresh dilution of the stocks for experimental use was prepared on the day of the measurements. The final concentration of DMSO was always kept below 0.5% v/v. Ouabain, tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), N6-cyclopentyladenosine (CPA), pertussis toxin and inorganic chemicals were purchased from Sigma Chemical (St. Louis, MO, USA). Phosphate-buffered saline (PBS) contained 140 mM NaCl, 5 mM KCl, 8 mM Na2HPO4, 3 mM NaH2PO4, pH 7.3.

Cell culture
The hamster vas deferens smooth muscle cell line (DDT1 MF-2) was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). DDT1 MF-2 cells were cultured at 37°C in humidified atmosphere of 5% CO2 and 95% O2. The growth medium was Dulbecco’s modified Eagle’s medium supplemented with 2 mM L-glutamine and 10% (v/v) fetal calf serum. Cells were collected by vigorous shaking of the flask, washed with PBS and kept at 2 × 107/ml concentration until they were used for flow cytometric measurements. To eliminate the endogenous adenosine, adenosine deaminase (2 E/ml) was added to the cells 10 min before the measurement. Cell viability was more than 90%, as assessed by the exclusion of trypan blue.

Flow cytometric determination of the membrane potential
The flow cytometric membrane potential measurements were carried out using the negatively charged bis oxonol dye that distributes across the membrane according to the Nernst equation. The protocol developed by Krasznai et al. (15) was used that allows the evaluation of the membrane potential in millivolts using fluorescence readings of the cells both in depolarized state and in the state of interest. For the measurements, a modified FACSTAR flow cytometer equipped with an argon ion laser was used (Becton Dickinson, Parsippany, NJ, USA). The oxonol fluorescence was excited by the 488-nm line at 200–400-mW power. The output optics contained a combination of a 520-nm longpass filter to block the scattered excitation light and a 540-nm band filter. Small angle forward-scattered light was used for electronic gating of the data collection allowing dead cell exclusion from the analysis. Cells were run at room temperature at a concentration of 105/ml. Measurement of the fluorescence histogram was completed after having reached equilibrium staining in about 2 min. Ligand-induced change in cell fluorescence was measured 3 to 5 min after addition of the appropriate ligand to the equilibrium sample, the time needed to reach a new equilibrium. To assess the modifying effect of a second ligand, it was added to the stained cell suspension in the second equilibrium. All experiments were repeated at least three times.

Electrophysiology
Whole cell membrane currents were recorded with Axopatch-200 and 200-A patch-clamp amplifiers in conjunction with Axon Instruments (Foster City, CA, USA) TL-1-125 and Digidata 1200 computer interfaces with varying sampling rates, applying low-pass filtering at half of the sampling frequency. Patch electrodes of 3–4 MΩ resistance were fabricated from GC150 F-15 borosilicate glass capillaries (Clark Electromedical Instruments, Reading, UK). The pipettes were filled with KF intracellular solution (140 mM KF, 2 mM MgCl2, 1 mM CaCl2, 11 mM EGTA and 10 mM HEPES, pH 7.22). The cells were bathed in a Ringer like (145 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2.5 mM CaCl2, 10 mM HEPES and 5.5 mM glucose, pH 7.35). The measured osmolarity of the solutions was 320 mOsm. Gigaseals in the order of 20–100 GΩ were formed by applying 50–70 H2Ocm negative pressure to the interior of the patch pipette. Subsequently, whole-cell measuring configurations were established by the application of negative pressure pulses. In most of the experiments the leak was negligible compared to the K+ currents. All patch clamp measurements were carried out at 20°C. Current determination protocols were started 12 min after entering whole cell configuration or later. K+ channel properties of the examined cells remained stable for up to several hours. During experiments 80% series resistance compensation was used. The adenosine receptor ligands were taken into the cells by perfusion. In order to demonstrate that adenosine-receptor ligands directly affect potassium currents, the cultured DDT1 MF-2 cells were incubated for 4 h in a cell culture media supplemented with 200 µg/ml pertussis toxin. Then the cells than were collected by vigorous shaking of the flask, washed with PBS and kept on ice until used for patch clamp experiments. The extracellular solution during patch clamp experiments also contained 200 µg/ml pertussis toxin.
Data analyses

For data acquisition and analyses, the pClamp6 program package (Axon Instruments, Inc.) was used. All experiments were repeated at least five times. Averaged data are indicated as the mean ± S.D. and were calculated using n experiments, where n indicates the number of independent experiments. Statistical analyses of differences between groups was carried out with Student’s t-test and a probability of 0.05 was taken as the level of statistical significance.

RESULTS

Flow cytometric measurements using the membrane potential sensitive fluorescent oxonol dye showed a rapid and maintained hyperpolarization of DDT1 MF-2 cells upon the administration of 50 nM CPA, a high affinity A1 adenosine receptor selective agonist (Fig. 1A). The effect of CPA (in a range of 10 to 3 μM) was immediate and the maximum hyperpolarization developed within 2 min. DPCPX (1 μM), a highly specific A1-receptor antagonist, significantly diminished the agonist-induced hyperpolarizing effect. Preincubation of the cells with 4-AP (100 μM), a potassium channel blocker, or DPCPX (1 μM), an adenosine A1-receptor antagonist, completely eliminated the CPA effect on the membrane potential, while preincubation with the Na+K+ ATP-ase blocker ouabain (5 μM) did not affect the CPA-induced hyperpolarization (not shown). Patch clamp experiments carried out on DDT1 MF-2 cells in the whole cell configuration showed an outward current with a reversal potential of −75 mV, which is equal to the potassium equilibrium potential. This current was completely and reversibly blocked by potassium channel blockers 4-AP (5 mM) or TEA (10 mM). Application of CPA in a range of 10 nM to 10 μM gradually and significantly increased this current with an approximately 30% increment at 1 μM concentration (n = 5) (Fig. 2A). Thus, the underlying potassium permeability change may be responsible for the hyperpolarization elicited by this A1-receptor agonist.

The A2A-receptor agonist CGS 21680 in the concentration range of 10 nM to 3 μM had an opposite effect on the membrane potential and potassium conductance of the DDT1 MF-2 cells as compared to that of the A1 agonist CPA. CGS 21680 in as low concentration as 50 nM significantly depolarized the cell membrane within 2 min (Fig. 1B). Subsequent addition of 1 μM CSC brought about a repolarization within 5 min adjusting the membrane potential very close to the control value. We could not detect significant CGS 21680 induced decrease of the transmembrane potential on cells preincubated with both xanthine-type (CSC, 1 μM) and non-xanthine type (ZM 241385, 1 μM) A2A adenosine receptor antagonists.
A

Receptor Ligands Regulate Membrane Potential

Also, CPA reverted the CGS 21680 induced depolarization of the cells (Fig. 1C). The reversal may be related to the four times higher expression of A₁ receptors compared to A₂A receptors by the DDT1 MF-2 cell (12). Patch clamp measurements showed a prompt decrease of the whole cell potassium current of the cells upon the administration of 10 nM to 10 μM CGS 21680 (n = 5). A representative voltage/current relationship is shown no Fig. 2B. The potassium channel blocking was completely abolished by washing out the A₂A agonist. The reversibility of the CGS 21680 mediated inhibition was also documented by the partial increasing of the current by the A₂A antagonist CSC (1 μM). The effect of CGS 21680 on the potassium current kinetics is shown on Fig. 3.

Fig. 2. Effect of adenosine receptor agonist on the potassium conductance of the membrane of DDT1 MF-2 smooth muscle cells. Peak values of whole cell K⁺ current in the presence of externally applied A₁ agonist CPA and A₂A agonist CGS 21680 are plotted in panels A and B, respectively, along with control values. Voltage steps lasting for 1000 ms were applied every 60 s from a holding potential of −120 mV in 10 mV increments from −70 to +20 mV. Selected traces are displayed on the inserts for test pulses of different amplitude registered from cells suspended in extracellular solution (upper set of traces) and from cells incubated in the presence of the agonists (lower set of traces). Measurement of whole cell K⁺ current started 2 min after the administration of the ligands. Leak current substraction has been applied. Data displayed are representative of five independent experiments.

B
CGS 21680 at 10 nM partially reduced the potassium current amplitude (Fig. 3A) but did not influence the inactivation of the current (Fig. 3B), while the application of higher concentration of the drug resulted in a significant inactivation of the current (n = 5). The inactivation has a peak at 100 nM CGS 21680 concentration (Fig. 3B). In order to demonstrate that the changes in potassium current are brought about by a direct coupling of the receptors with the channel apparently not involving G proteins, the experiment with the most effective concentration of CGS21680 (100 nM) were repeated using pertussis toxin-treated cells. There were no significant differences obtained.

Fig. 3. Effect of A<sub>2A</sub> agonist CGS 21680 on the potassium conductance of membranes of DDT1 MF-2 smooth muscle cells. During whole cell K<sup>+</sup> current recording, the cells were first perfused by the Normal Ringer control extracellular solution (NR) described in Materials and Methods. When both K<sup>+</sup> current amplitude and kinetics stabilized, the perfusion system was switched to CGS 21680 dissolved in NR at different concentrations ranging from 10 nM to 7.5 µM, and the new equilibrium of current was observed. The alterations evoked by CGS 21680 treatment show a concentration dependence having the highest effect at 100 nM. The above stand for both the remaining fraction of peak current represented by Panel A or the acceleration of inactivation shown by Panel B (n = 5). Current levels were measured when depolarizing pulses were applied from a holding potential of −120 mV to +50 mV for 2000 ms every 15 s. Inactivation time constants were determined by single exponential fitting. Error bars represent standard deviations.

Fig. 4. Effect of A<sub>2A</sub> adenosine receptor agonist CGS 21680 in the presence of pertussis toxin on the potassium conductance of the membrane of DDT1 MF-2 smooth muscle cells. Peak values of whole cell K<sup>+</sup> current in the presence of externally applied A<sub>2A</sub> agonist CGS 21680 (50 nM) are plotted in panel A and B, respectively, along with control values. Depolarization pulses lasting for 100 ms from a holding potential of −120 mV to +40 mV were applied in every 60 s. Selected traces are displayed. Upper set of traces (A) show the currents of the control cells and lower set of traces (B), the currents of the cells incubated for 4 h with 200 µM pertussis toxin. During the measurements, the pertussis toxin was present in the external solution. Measurement of K<sup>+</sup> current started 2 min after the administration of the ligand. Leak current substraction has been applied. Data displayed are representative of five independent experiments.
neither in the remaining fraction of the peak current of the pertussis toxin (200 µg/ml, incubated for 4 h)-treated cells compared to the control cells (73 ± 12%, n = 5 and 66.9 ± 8.8%, n = 5, respectively) (Fig. 4) nor in the change of the inactivation kinetics of the current (54 ± 9.7%, n = 5 and 64.8 ± 12%, n = 5, respectively).

**DISCUSSION**

Our flow cytometric data and the related patch clamp measurements demonstrate that the binding of A<sub>1</sub>-specific agonist CPA hyperpolarizes the DDT1 MF-2 cells, while binding of the A<sub>2A</sub> agonist CGS 21680 induces depolarization. The effect of both agonists can be decreased or abolished completely by the appropriate antagonists depending on the concentrations applied.

To our best knowledge, this is the first report on the modulation of potassium current in DDT1 MF-2 cells by selective P<sub>2</sub> adenosine receptor activators. Molleman et al. (16) failed to detect any action of high dose of adenosine (1 mM) on membrane current or the ATP-evoked membrane currents in this cell line. However, these findings do not directly contradict to our results, as adenosine may bind to P<sub>2</sub> receptors and, in addition, the weak binding of this ligand to both A<sub>1</sub> and A<sub>2</sub> receptors may induce various receptor activation processes resulting in an insignificant effect. In the present study, the actions of subtype-specific agonists were analyzed; therefore, the effects could be manifested separately.

Responsiveness of potassium channels to various subtype-specific adenosine analogues varies from tissue to tissue. Although both A<sub>1</sub> and A<sub>2</sub> receptors are present in smooth muscle cells from the pig coronary artery, occupation of A<sub>1</sub> adenosine receptor activates potassium current, but that of A<sub>2A</sub> does not (17). In arterial myocytes, only A<sub>2A</sub>-receptor agonist was able to activate potassium channels, whereas A<sub>1</sub> activators proved to be indifferent (18). In bovine adrenal zona fasciculata cells, the expressed non-activating potassium current could be selectively inhibited by both A<sub>1</sub>, A<sub>2A</sub> or A<sub>1</sub> adenosine receptor agonists, and these inhibitions were associated with membrane depolarization (19). According to our recent findings, DDT1 MF-2 cells are unique in their ability to respond with increased potassium conductance and hyperpolarization to A<sub>1</sub>-receptor activation and decreased potassium current and depolarization to A<sub>2A</sub>-receptor activation. Therefore, these cells could be useful pharmacological tools to analyze the A<sub>1</sub>- and A<sub>2A</sub>-receptor-mediated mechanisms in the same cell line.

It has been shown recently that a close relation exists between membrane potential and adenylyl cyclase activity (20). Beltran et al. (21) showed that the membrane potential directly modulates the adenylyl cyclase activity in sea urchin sperm, which may have a role in the induction of sperm motility. A direct relation between the membrane potential and adenylyl cyclase activity was also demonstrated by Izumi et al. (22). Reddy et al. (23) reported on a synergistic stimulation of adenylyl cyclase activity in cultured cerebellar neurons by depolarization. They hypothesized that conformational changes in the catalytic subunit of the enzyme caused by depolarization may enhance stimulation of adenylyl cyclases by the guanyl nucleotide stimulatory protein. They also showed that this phenomenon was Ca<sup>2+</sup>-independent and not attributable to the regulation by intracellular Ca<sup>2+</sup>. This finding is consistent with our observation on the CGS 21680-induced depolarization and the reported adenylyl cyclase stimulation by receptor binding of A<sub>2A</sub> agonists. Our CPA data also match the assumption that the membrane potential dependence of the adenylyl cyclase activity under hyperpolarizing conditions is still the same as Reddy et al. (23) reported for the physiological to depolarized membrane potential range.

We suggest that stimulation or inhibition of the adenylyl cyclase by A<sub>2A</sub>- and A<sub>1</sub>-receptor activation, respectively, can occur by a mechanism involving a ligand-induced change in the transmembrane potential among the first elementary steps as alterations in the membrane polarity may lead to voltage-dependent conformational change of the enzyme. We also observed that A<sub>1</sub> agonist might modulate the membrane potential changes induced by A<sub>2A</sub> agonist. This interaction between A<sub>2A</sub> and A<sub>1</sub> receptors may serve as a basis for a complex regulation of the adenylyl cyclase realized by binding of any pair of A<sub>2A</sub>- and A<sub>1</sub>-specific agonists and antagonists from a large number of physiological ligands to the appropriate adenosine receptors. The ratio of the expressed A<sub>1</sub> and A<sub>2</sub> type of receptors is tissue-specific and the kind and concentration of the available agonists and antagonist may also vary from tissue to tissue. This variability together with the diversity of the possible cAMP-signal-driven specific processes explain why the manifestations of the adenosine receptor activation may be so different in the various organs and tissues. The outlined mechanism for regulation of adenylyl cyclases then generates cAMP signals that may contribute to various modulatory events.

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