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P_2-Purinoceptors in a renal epithelial cell line (LLC-PK_1)

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Extracellular ATP and ADP interact with cell surface receptors to regulate a number of physiological processes, which include increases in membrane permeability, relaxation or contraction of various smooth muscles, platelet aggregation, and modulation of neuronal excitability [1, 2]. Two major classes of purinoceptors have been identified: P_1-receptors which are activated by adenosine, and P_2-receptors which are activated by ATP and ADP, but not by AMP or adenosine [3]. In isolated aortic endothelial cells ATP induces a biphasic increase in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), an increase which consists of a rapid and transient and a subsequent sustained phase [4]. The transient increase in $[Ca^{2+}]_i$ is caused by the release of calcium from intracellular stores, while the sustained increase is probably mediated by the activation of Ca^{2+} channels on plasma membranes. The kidney is composed of heterogeneous nephron segments and a variety of cell types, including arterial endothelial cells. Hence, the use of cell culture techniques has permitted the study of a relatively homogeneous preparation and has offered advantages for the examination of many renal cell functions. In the present study we investigated the localization of P_2-purinoceptors in renal non-vascular cells, LLC-PK_1, which have been used as a model for the proximal tubular epithelium [5]. To demonstrate whether LLC-PK_1 cells respond to ATP binding with modulation of intracellular calcium, we examined the effect of ATP on $[Ca^{2+}]_i$.

Materials and Methods

Cell culture. The LLC-PK_1 cells, obtained from the American Type Culture Collection (ATCC CRL-1392), were grown to confluence (about 5×10^5 cells/cm^2) in 199-Earle medium supplemented with 10% fetal bovine serum. The cells were subcultured every 5–7 days using 0.02% EDTA and 0.05% trypsin in phosphate-buffered saline. For fluorometric experiments, the cells were grown on glass coverslips (7 × 13 mm; thickness, under 0.12 mm; Matsunami Glass Ind., Ltd., Osaka, Japan).

Binding experiments. LLC-PK_1 monolayers on 24-well tissue culture plates (Corning) were washed twice with binding assay medium [0.1% gelatin, 116 mM NaCl, 5.4 mM KCl, 1.0 mM NaH_2PO_4, 0.2 μM $Fe(NO_3)_3$, 5.6 mM dextrose, 5 mM $NaHCO_3$, and 10 mM Hepes, pH 7.4]. The monolayers were incubated in a final volume of 0.3 mL of binding assay medium. The reaction was initiated by the addition of radioactive-labeled adenine nucleotides. Non-specific binding was defined as the amount of ATP bound in the presence of 10 μM non-radioactive ATP. After different time intervals the reaction was terminated by rapidly aspirating the incubation medium and washing the monolayers three times with 1 mL of the ice-cold binding assay medium. The cells were then disintegrated with 0.3 mL of 0.2 N NaOH, and the radioactivity was measured by liquid scintillation counting.

Intracellular free Ca^{2+} measurements. Intracellular free Ca^{2+} was measured using a fluorescent probe, fura 2, essentially as described [6]. The LLC-PK_1 monolayers were placed into a thermostatted cuvette in a Hitachi F-2000 spectrofluorometer, and incubated with a 2 μM concentration of the acetoxymethyl ester of fura 2 (Dojindo Lab., Kumamoto, Japan) with 0.01% Cremophor EL (a non-cytotoxic detergent) for 30 min at 30° in assay medium [116 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 0.2 μM $Fe(NO_3)_3$, 5.6 mM dextrose, 5 mM $NaHCO_3$, and 10 mM Hepes, pH 7.4]. The indicator-loaded monolayers were perfused with assay medium at a flow rate of 0.3 mL/min. After washing the surplus indicator, the Ca^{2+}-dependent emission intensity from the cells was recorded continuously at excitation wavelengths of 340 and 380 nm alternately and an emission wavelength of 510 nm. Calibration of fura 2 fluorescence was performed as previously described [7]. Maximum and minimum fluorescence values were recorded at 10^{-3} and 10^{-9} M Ca^{2+}, respectively, by lysing the cells in 0.1% Triton X-100 followed by chelation of free Ca^{2+} on addition of 5 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA). The values of $[Ca^{2+}]_i$ were calculated using an effective dissociation constant of 224 nM for Ca^{2+} binding to fura 2.

Results and Discussion

The specific binding of [^{3}H]ATP to LLC-PK_1 monolayers was temperature dependent. At a temperature of 30° the specific binding reached an equilibrium value within 15 min and was stable up to 60 min. At 4°, the specific binding did not reach an equilibrium within 60 min, so we performed binding experiments at 30° for 15 min. Moreover all binding experiments were performed in Mg^{2+}- and Ca^{2+}-free medium since the elimination of Mg^{2+} and Ca^{2+} from the medium was beneficial in that it significantly reduced hydrolysis and uptake of adenine nucleotides.

Adenosine and adenine nucleotides inhibited [^{3}H]ATP binding in LLC-PK_1 monolayers in a concentration-dependent manner (Fig. 1a). The order of potency observed was ATP = ADP > AMP > adenosine, which is consistent with that for P_2-purinoceptors. Moreover, a similar inhibition by adenosine and adenine nucleotides with relative potency was also observed in experiments utilizing [^{35}S]adenosine 5′-(β-thio)diphosphate (ADPβS) as a radioligand for binding (Fig. 1b). ADPβS is a selective P_2-agonist and much less sensitive to hydrolysis compared to other ATP and ADP analogs [8]. These data strongly suggest the presence of P_2-purinoceptors in LLC-PK_1 cells. Scatchard analysis of [^{35}S]ADPβS binding to LLC-PK_1 monolayers gave a K_d of 90.5 nM and a B_{max} of 1.14 pmol/10^6 cells (Fig. 2).

Figure 3a shows a typical $[Ca^{2+}]_i$ change experiment observed upon stimulation with 10 μM ATP. Intracellular

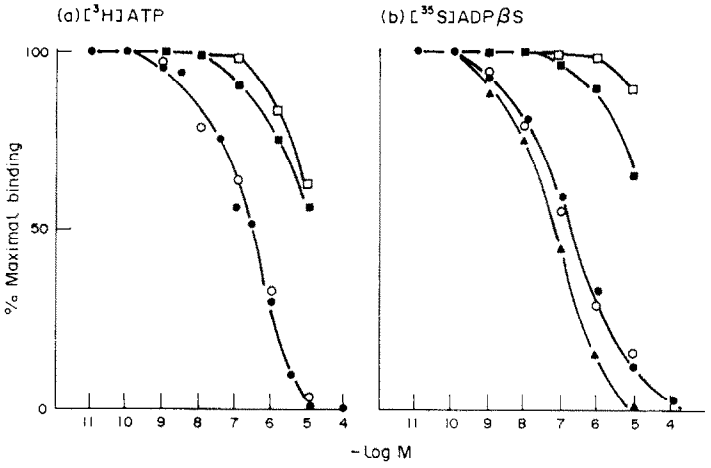

Fig. 1. Concentration-dependent inhibition of [^{3}H]ATP or [^{35}S]ADPβS binding by adenosine and adenine nucleotides. LLC-PK$_1$ monolayers were incubated as described in Materials and Methods with [^{3}H]ATP (5 nM) (a) or [^{35}S]ADPβS (5 nM) (b) for 15 min at 30° in the presence of various concentrations of ATP (●), ADP (○), AMP (■), adenosine (□), and ADPβS (▲). Data are expressed as a percent of maximal binding. The 100% values for [^{3}H]ATP and [^{35}S]ADPβS were 84.0 ± 2.6 and 49.8 ± 2.0 fmol/10^6 cells, respectively. Each curve represents the mean of three similar experiments.

free Ca^{2+} increased from a quiescent level of about 90 nM to a peak of 350 nM, and then declined slowly. The plateau level (about 200 nM) was achieved for 5–20 min after the addition of ATP. On the other hand, Fig. 3b shows the $[Ca^{2+}]_i$ change monitored in Ca^{2+}-free buffer containing 0.1 mM EGTA. Although the rapid and transient phase remained, in response to ATP, $[Ca^{2+}]_i$ decreased more rapidly and reached the quiescent level within 10 min. Thus, the rapid and transient increase in $[Ca^{2+}]_i$ is considered to be induced by the discharge of Ca^{2+} from the intracellular store site and the sustained increase is by Ca^{2+} influx across the plasma membrane. ADPβS also increased in $[Ca^{2+}]_i$ in a quantitatively similar manner (data not shown). Recently, Nanoff *et al.* [9] demonstrated that P_2-purinoceptors stimulate the formation of inositol phosphates in rat renal cortex slices. Therefore, the rapid and transient release of Ca^{2+} from intracellular stores is probably induced by activation of phosphoinositide breakdown.

The LLC-PK$_1$ cells have been characterized as a model system for the analysis of epithelial function in the proximal tubules [5]. Recently it has been shown that vasopressin, oxytocin [10] and endothelin [11] induce a transient mobilization of $[Ca^{2+}]_i$ in these cells. Therefore, these cells

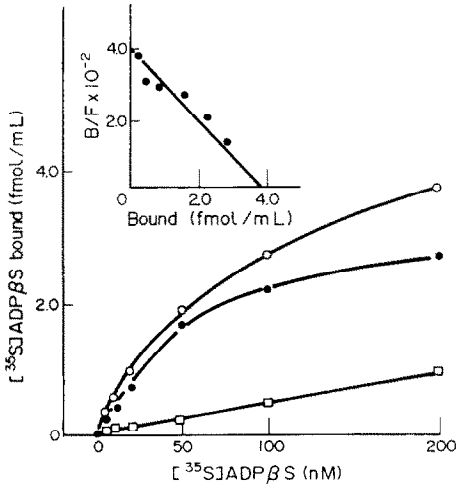

Fig. 2. Saturation equilibrium binding of [^{35}S]ADPβS. LLC-PK$_1$ monolayers were incubated for 15 min at 30° as described in Materials and Methods with [^{35}S]ADPβS at the concentrations indicated, with (□) or without (○) 10 μM ADPβS. Specific binding (●) is the difference between the two curves. Mean values of triplicate determinations are presented.

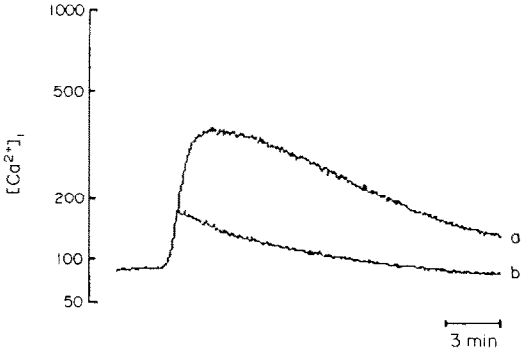

Fig. 3. ATP-induced $[Ca^{2+}]_i$ changes in LLC-PK$_1$ monolayers. LLC-PK$_1$ monolayers were grown to confluence on coverslips, loaded with fura 2, and perfused with assay medium (a) or Ca^{2+}-free assay medium containing 0.1 mM EGTA (b). The $[Ca^{2+}]_i$ change was measured fluorometrically at 30° as described in Materials and Methods. ATP (10 μM) was added at the time indicated by the arrow. The traces are representative of at least three similar experiments.

may be useful as a model system for studying not only the direct effect of extracellular ATP but also the heterologous regulation coupled to calcium signalling of renal tubular cell function.

The present study has demonstrated that there are specific receptors for extracellular ATP and ADP in renal epithelial cells, and that the binding of ATP induced the biphasic increase in $[Ca^{2+}]_i$, which consists of an initial and a second phase. The transient increase in $[Ca^{2+}]_i$ is considered to be caused by the release of calcium from the intracellular Ca^{2+} pool and the sustained increase may be induced by the influx from the extracellular buffer.

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Glucuronidation of imipramine in rabbit and human liver microsomes: assay conditions and interaction with other tertiary amine drugs

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Direct conjugation of nitrogen atoms in drugs with the C-1 of glucuronic acid has been observed for a number of different amines [1]. Of particular interest is the formation and urinary excretion of quaternary ammonium glucuronides from tertiary amine drugs such as cyproheptadine [2, 3], tripelennamine [4], cyclobenzaprine [5] and chlorpromazine [6]. This pathway of excretion was originally thought to be unique to the higher primates (e.g. man, chimpanzees), with most laboratory animals apparently unable to excrete these conjugates [3, 5], however more recent reports have demonstrated the excretion of quaternary ammonium glucuronides of the imidazole antifungal agents tioconazole and croconazole and the anti-anaphylactic ketotifen in the rabbit [7–9]. In addition to *in vivo* studies, it has been shown that both human and rabbit (but not rat) liver can form *N*-glucuronides *in vitro* [8, 10–12]. However, the analytical methods generally associated with the investigation of these reactions have until now been cumbersome and time consuming, requiring specialist analysis such as mass spectrometry (e.g. Refs 10 and 11), thereby severely limiting the investigation of the uridine diphosphoglucuronosyltransferase(s) (UDPGT*) [13] involved in these reactions.

We have therefore developed a radiometric assay procedure for determining the microsomal glucuronidation of the tricyclic antidepressant imipramine which offers considerable advantages over other procedures used to measure the formation of quaternary ammonium glucuronides, such as HPLC and mass spectrometry.

* Abbreviations: UDPGT, uridine diphosphoglucuronosyltransferase; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid.

Materials and Methods

Imipramine hydrochloride cyproheptadine hydrochloride, ketotifen (fumarate salt), cyclizine hydrochloride,