ELSEVIER

European Journal of Pharmacology 329 (1997) 245–252

ejp

Indomethacin and piroxicam inhibit Na^+-adenosine transport in rat renal brush-border membranes

Carmen González-Castillo [a], Martha Franco [b], Alicia Quintana [a], Bruno Escalante [c], Flavio Martínez [a,*]

[a] *Department of Pharmacology, Facultad de Medicina de la Universidad Autónoma de San Luis Potosí, Avenida Carranza 2405, 78210-Los Filtros, SLP, México*

[b] *Department of Nephrology, Instituto Nacional de Cardiología, Juan Badiano 1, 14080-Tlalpan, DF, Mexico*

[c] *Department of Pharmacology-CINVESTAV, Avenida IPN 2508, 07300-Zacatenco, México City, Mexico*

Received 14 October 1996; revised 17 April 1997; accepted 22 April 1997

Abstract

The effects of the cyclooxygenase inhibitors, indomethacin and piroxicam, were evaluated on Na^+-dependent [^{3}H]adenosine transport in rat renal brush-border membranes of the outer renal cortex of the rat. Adenosine co-transport (1–10 μM) was estimated in the presence of 0.001–10 μM indomethacin and piroxicam. Both drugs inhibited the Na^+-dependent transport in a dose-dependent manner with IC_{50} of 3.5 μM and 0.1 μM, respectively. The Na^+-independent transport was not modified. Preincubations carried out on the vesicles with 10–50 μM arachidonic acid increased transport in a dose-dependent manner up to 1.7 times. Whereas 50 pM to 5 μM prostaglandin E_2 in the presence of indomethacin did not change carrier activity, 5 μM prostaglandin E_2 increased the Na^+-dependent transport 1.5 times. Other prostanoid synthesis pathways were investigated with 10 μM nordihydroguaiaretic acid (lipoxygenase inhibitor), and 17-octadecynoic acid and clotrimazole (leukotriene and cytochrome P_{450} inhibitors). Our results demonstrated that the Na^+-dependent adenosine transport in brush-border membranes was inhibited by indomethacin and piroxicam, suggesting that cyclooxygenase activity might modulate this co-transport. © 1997 Elsevier Science B.V.

Keywords: Adenosine transport, Na^+-dependent; Brush-border membrane vesicle; Cyclooxygenase; Prostanoid; Non-steroidal anti-inflammatory drug (NSAID)

1. Introduction

Na^+-coupled transport mechanisms in the kidney tubules induce a net reabsorption flux of substrates and are under hormonal control (Yusufi et al., 1985; Gesek and Schoolwerth, 1990) and subject to autacoid receptor activation (Siragy et al., 1989; Friedlander et al., 1990). Among the Na^+-dependent co-transport systems, a Na^+-coupled adenosine transporter has been characterized in the luminal membrane of the proximal tubule of rat kidney (Le Hir and Dubach, 1984; Trimble and Coulson, 1984; Franco et al., 1990). The physiological relevance of this carrier is not clear nor is it clear whether this transporter is under hormonal or autacoid regulation.

Cyclooxygenase lipoxygenase and cytochrome P_{450} arachidonic acid derivatives are produced in the epithelial tubular cells in response to hormonal stimulation and, through intracellular signaling, regulate transepithelial ion and water transport (Smith, 1992; Hébert et al., 1991; Escalante et al., 1991; Aarab et al., 1993). Infusion of arachidonic acid induces decreased Na^+ reabsorption in the proximal tubule of rabbit kidney through prostaglandin I_2 production (Kinoshita et al., 1989). Cytochrome P_{450} arachidonate metabolites, 20-hydroxyeicosatetraenoic acid and 20-carboxyarachidonic acid, inhibit ^{86}Rb transport in Henle's thick ascending limb of the rabbit kidney (Escalante et al., 1991). Prostaglandin E_2 directly modulates antidiuretic hormone effects inhibiting water and Na^+ transport in isolated cortical tubules (Hébert et al., 1991) and in renal epithelial cells in culture (Martínez and Reyes, 1984). In addition, prostaglandin E_2 in Madin-Darby canine kidney cells inhibits Na^+/K^+-ATPase and these effects are blocked by cyclooxygenase inhibitors (Cohen-

* Corresponding author. Tel.: (52-48) 130-499, ext. 26; Fax: (52-48) 176-976; e-mail: martinej@deimos.tc.uaslp.mx

PII S0014-2999(97)00155-6

Luria et al., 1994). Thus, prostaglandin E_2 might participate in the modulation of other transport mechanisms in renal brush-border membrane vesicles.

The aim of this work was to evaluate the role of the cyclooxygenase pathway in Na^+-dependent adenosine transport in renal brush-border membrane vesicles of the outer cortex of the rat kidney.

2. Materials and methods

2.1. Preparation of brush-border membrane vesicles

The preparation of vesicles was carried out at 4°C according to a modification of the Booth and Kenny method (Booth and Kenny, 1974; Martínez et al., 1990). Under pentobarbital Na^+ anesthesia, the kidneys of 10 rats were removed and chilled in an ice-cold isolation buffer (300 mM mannitol, 12 mM Tris-H_2SO_4, pH 7.4) immediately after being excised. The outer cortex (1 mm thick) was dissected, minced, weighed and suspended in a 20-fold volume of hypotonic homogenization buffer (100 mM mannitol, 12 mM Tris-H_2SO_4, pH 7.4). Portions of outer cortex (8.0 g) were homogenized for 2.5 min in 160 ml of homogenization buffer with a SDT tissumizer homogenizer (Tekman, model TR-10 Germany) on setting 7, for 1 min. A sample of this homogenate was withdrawn for protein and enzyme determinations. After the addition of 10 mM $MgCl_2$, the homogenate was stirred for 15 min on ice, and then centrifuged at $1900 \times g$ for 12 min in a SS34 rotor, Sorvall RC-5B centrifuge (DuPont Instruments). The supernatant was centrifuged at $20000 \times g$ for 12 min. The pellet was resuspended in 60 ml of the homogenization buffer with a Dounce glass pestle (10 strokes) followed by the addition of 10 mM $MgCl_2$; after 15 min the suspension was centrifuged again at $20000 \times g$ for 12 min. The pellet was suspended in 20 ml of intravesicular buffer pH 7.4 containing 300 mM mannitol, 20 mM HEPES-Tris and 50 μM EHNA, an adenosine deaminase inhibitor; it was homogenized by hand (10 strokes) with a Dounce glass pestle homogenizer, left to equilibrate for 60 min and centrifuged at $30000 \times g$ for 20 min. This step was repeated, and the final pellet was suspended in intravesicular buffer by aspirating the suspension with a syringe through a 25-gauge needle (10 times). The final volume of brush-border membrane vesicle suspension was adjusted to yield a protein content of 35–50 mg/ml. The vesicles were frozen and stored in liquid nitrogen until required.

The purity of the prepared membrane vesicles was monitored by measuring the specific activity of leucine aminopeptidase (a typical brush-border enzyme) and Na^+/K^+-ATPase (basolateral marker enzyme) in the homogenate and in the final vesicle preparations as previously reported (Martínez et al., 1990). Compared with the initial homogenate, enzyme activity was enriched by a factor of 11.26 ± 0.59 for leucine aminopeptidase (mean $\pm$ S.E., $n = 9$) and 1.08 ± 0.11 for Na^+/K^+-ATPase (mean $\pm$ S.E., $n = 9$).

2.2. Protein determination

Protein content was estimated according to the bicinchoninic acid method (Smith et al., 1985). The curve was adjusted with IgG as the standard protein between 1 and 20 μg/ml. Samples were 50 μl of the appropriately diluted brush-border membrane vesicles (1:100, v/v) plus 950 μl of the working solution, incubated at 60°C for 30 min. Readings were carried out at 562 nm in a Shimadzu Spectrophotometer (UV-1601).

2.3. Transport experiments

Before the transport experiments, brush-border membrane vesicles were thawed at 25°C and diluted with intravesicular buffer in order to have 35–60 μg of protein per sample/filter. The vesicles were rehomogenized by passing them 20 times through a 25-gauge needle with a syringe and kept on ice until the beginning of the experiment. When the effects of the inhibitors, arachidonic acid and prostanoids were evaluated, membrane suspensions were preincubated for 60 min for enzyme inhibitors, 1–5 min for arachidonic acid and 10 min for prostaglandin E_2.

Adenosine transport was determined with the rapid filtration technique. The reaction was initiated by mixing 20 μl of brush-border membrane vesicles with 100 μl of uptake medium. The Na^+-dependent adenosine transport was evaluated with a Na^+ buffer: 100 mM mannitol, 100 mM NaCl, 20 mM HEPES-Tris, pH 7.4; the Na^+-independent adenosine transport was determined with Na^+ replaced by K^+: 100 mM mannitol, 100 mM KCl, 20 mM HEPES-Tris, pH 7.4. Labeled [^{3}H]adenosine (10 μCi/ml) and non-labeled adenosine were added to the uptake media in order to obtain adenosine concentrations from 0.5 to 10 μM. After appropriate time intervals, 5, 30, 60 and 120 s, the reaction was terminated by addition of 1 ml of ice-cold stop solution (300 mM mannitol, 80 mM Na_2SO_4, 5 mM Hepes-Tris, 10 μM dypiridamole, pH 7.4). The mixed solution was then poured immediately on to a moistened filter, kept under suction in a Millipore device (XX2702550).

The filter was then washed twice with 1 ml of ice-cold stop solution. Filtration was completed in 2–3 s. The filters were then removed and placed in a scintillation vial with 5 ml of scintillation fluid (Aquasol 2, NEN-DuPont). The radioactivity remaining on the filters was measured in a liquid scintillation counter (1209 Wallac Rackbeta). Blanks were subtracted and values were corrected for non-specific filter binding of radiolabeled solutes in the absence of membrane vesicles. The appropriate transformations of dpms to mol were made for each experiment and protein content. Results are expressed in mol/mg protein.

2.4. Determination of adenosine metabolism in membrane preparations

Before initiating the studies of adenosine transport, it was necessary to make sure that the adenosine added during uptake was not metabolized during the incubation time. Labeled adenosine was estimated in brush-border membrane vesicles with a microassay method for enzymes of purine metabolism (Kisaki and Sakurada, 1977): cellulose acetate membranes (Cellogel, Chemetron Italy) soaked in citrate buffer (0.1 M sodium citrate, pH 3.4) were placed in a LKB-Bromma (2117-Multiphor) electrophoretic chamber. 6 μl of standard 5 mM solutions of non-labeled adenosine, inosine, ATP, ADP, AMP were used as carriers for the radioactive solution.

20 μl of membrane preparations was incubated with 100 μl of the following solutions 100 mM mannitol, 100 mM NaCl or 100 mM KCl, 20 mM HEPES, pH 7.4, and 1 μM [^{3}H]adenosine, at 5, 60 and 3600 s. The reaction was stopped with 500 μl perchloric acid and centrifuged at 59 600 × g in a Beckman centrifuge (L8-50 M/E) at 4°C for 20 min.

The supernatant was withdrawn and stored in liquid nitrogen until required. The electrophoresis pattern was obtained by applying a 10 μl aliquot at the origin on a Cellogel membrane surface, and then separated at 100 V for 3 h. The marker bands were identified under ultraviolet light, cut out and immersed in 5 ml of scintillation liquid (Aquasol 2, NEN-Dupont). The standard solutions had previously been separated, under the same conditions, to identify each metabolite according to its migration pattern. Uric acid was identified in a similar manner but using a different buffer (100 mM-Tris HCl, pH 7.4). The results are expressed as percentages of the initial label.

When 1 μM [^{3}H]adenosine and the vesicles were assayed the recovery after 5 s was 85 ± 15% adenosine and 10 ± 2% inosine; at 30 s adenosine recovery was 65 ± 15% and inosine 25 ± 5%; at 60 min adenosine recovery was 45 ± 15% and those of inosine, hypoxanthine and uric acid were 30 ± 15%, 5 ± 2% and 25 ± 5%, respectively.

2.5. Prostaglandin E_2 determination

Prostaglandin E_2 was determined with an enzyme immunoassay kit (#514010, Cayman). Brush-border membrane vesicles were first incubated with either 10 μM indomethacin or 50 μM arachidonic acid during the appropriate time intervals. Samples were 50 μl of diluted (1:100, v/v) membrane supernatants. Determinations were performed in duplicate in two different membrane batches. Results were expressed as pg/mg protein.

2.6. Statistical analysis

The experimental data are reported as means ± S.E. ($n = 6$). Experiments were repeated at least 6 times with different membrane preparations and each point represents experiments in triplicate. Michaelis-Menten kinetics and their respective constants were calculated by non-linear regression analysis with the appropriate equation in the computer program Enzfitter (Elsevier-Biosoft, UK). IC_{50} values for inhibitory drugs were obtained by Probit analysis. The results were analyzed with the Sigma Stat computer program (Jandel, USA). Statistical differences with $P < 0.05$ were considered significant. Student's t-test was used to analyze differences between paired groups. An analysis of variance (ANOVA) was used to compare differences in the presence of drugs, followed by a Dunnett's test for multiple comparisons.

2.7. Materials

[2-^{3}H]Adenosine (20.0 Ci/mmol, 740 GBq/mmol) was purchased from Amersham (UK). Scintillation fluid, Aquasol-2 (NEF-952) was obtained from Du Pont (USA). The bicinchoninic acid protein assay kit was purchased from Pierce (USA). Adenosine:9-β-D-ribofuranosyl adenine, *erythro*-9-(2-hydoxy-3-nonyl)-adenine, indomethacin:(1-[*p*-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid) and immuno-γ-globulin were purchased from Sigma (St. Louis, MO, USA). Dipyridamole was purchased from Research Biochemicals International (USA). Cellulose acetate type 0.65 μm pore size filters (DAWP 025 00) were obtained from Millipore (USA). Arachidonic acid, prostaglandin E_2, clotrimazole (1-[*o*-chloro-α,α-diphenyl]imidazole),nordihydroguaiaretic acid, 17-octadecynoic acid and prostaglandin E_2 enzyme immunoassay kit were obtained from Cayman (Ann Arbor, MI, USA). All other chemicals were obtained from Merck (Germany) and were of analytical grade.

3. Results

Initially the kinetic data for the Na^+-dependent adenosine transport were obtained for 1–10 μM adenosine concentrations: $K_m = 2.67 \pm 0.53$ μM and $V_{max} = 211.6 \pm 14.7$ pmol/mg protein × min (data not shown).

To assess the effect of cyclooxygenase inhibitors on adenosine transport we evaluated the Na^+-dependent adenosine transport in the presence and absence of indomethacin. Since cyclooxygenase inhibitors require several minutes of incubation to achieve maximal inhibition of enzyme activity in vitro (Ouellet and Percival, 1995), brush-border membranes were incubated 60 min in the presence of 10 μM indomethacin or piroxicam prior to the initiation of adenosine transport experiments. Under these conditions either indomethacin (Fig. 1A) or piroxicam (Fig. 1B) abolished the Na^+-dependent adenosine transport at all times. However, when 10 μM indomethacin was added at time zero (without previous incubation), it induced a minor inhibition of Na^+-dependent adenosine

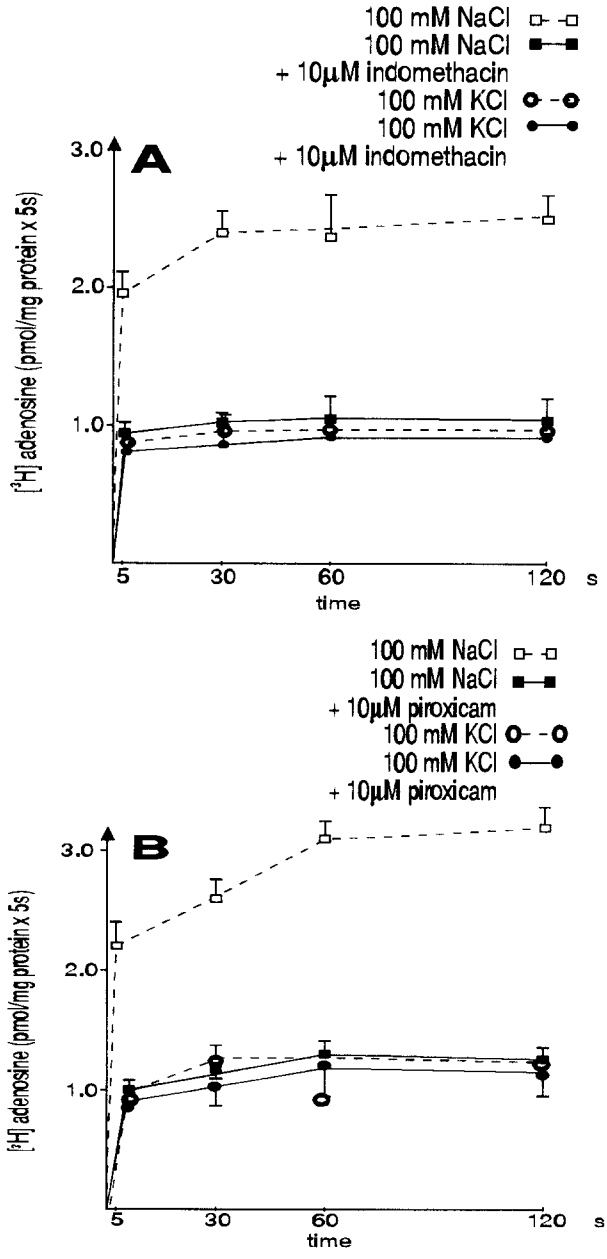

Fig. 1. Effect of indomethacin and piroxicam on the time course of [^{3}H]adenosine transport in rat renal brush-border membrane vesicles. 1 μM [^{3}H]adenosine transport was evaluated at 5, 30, 60 and 120 s in the presence of an outside > inside-directed 100 mM NaCl (□) and 100 mM KCl (○) gradient. The effects of indomethacin (A) and piroxicam (B) were evaluated in membranes treated with 10 μM of these drugs for 60 min before the experiment was started ($t = 0$) in the presence of 100 mM NaCl (■) or KCl (●). Data are expressed as the means ± S.E., $n = 6$.

transport, which was significant only at 5 s (2.51 ± 0.16 control vs. 1.68 ± 0.12 pmol/mg protein, indomethacin). The rest of the time course remained unchanged and Na^+-independent adenosine transport was not affected.

Under similar incubation conditions (60 min) indomethacin failed to inhibit the time course of Na^+-dependent [^{14}C]leucine transport. This was also observed with piroxicam on Na^+-dependent [^{14}C]lactate transport.

Both indomethacin and piroxicam inhibited Na^+-dependent adenosine transport in a concentration-dependent manner. In the 0.001–10 μM range of concentrations, indomethacin (Fig. 2A) and piroxicam (Fig. 2B) inhibited 5-s Na^+-dependent adenosine transport. Maximal inhibition was attained with 10 μM for both drugs. IC_{50} values

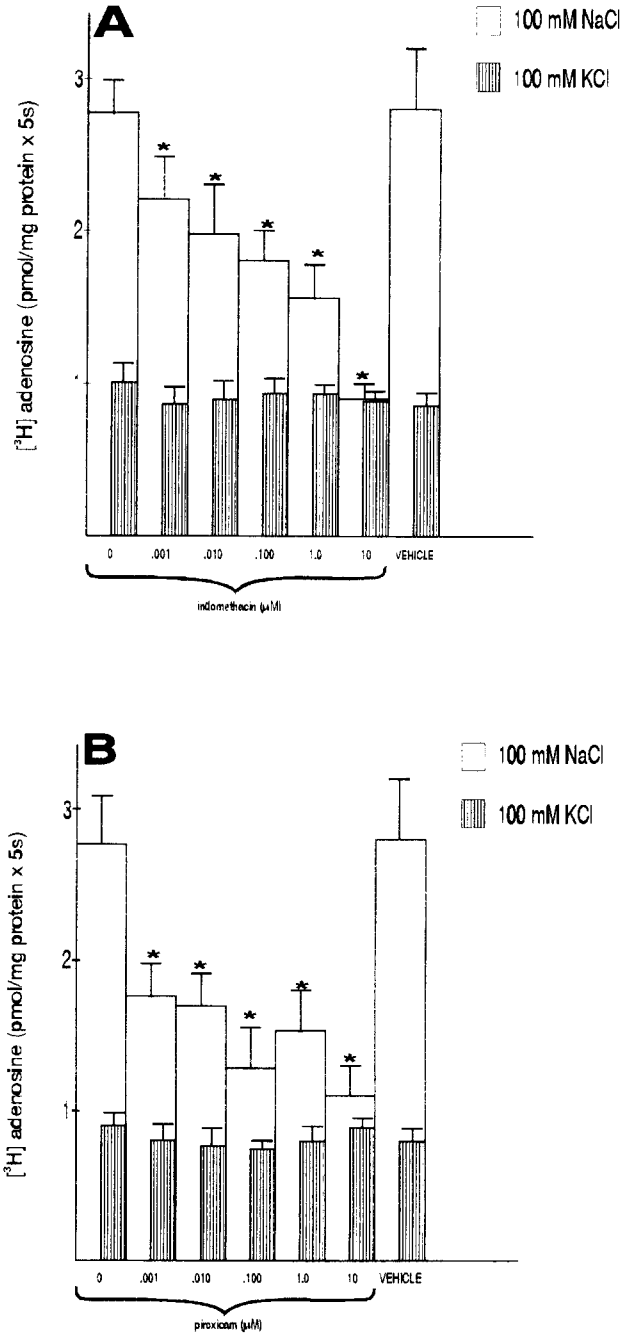

Fig. 2. Dose-dependent effects of indomethacin and piroxicam on [^{3}H]adenosine transport. Brush-border membrane preparations were preincubated for 60 min prior to uptake determinations. The effects of 0.001–10 μM indomethacin (A) and piroxicam (B) were evaluated at 5 s in the presence of an outside > inside-directed 100 mM NaCl (open bars) and KCl (hatched bars) gradient. Data are expressed as the means ± S.E., $n = 6$, * $P < 0.05$, NaCl + indomethacin vs. control.

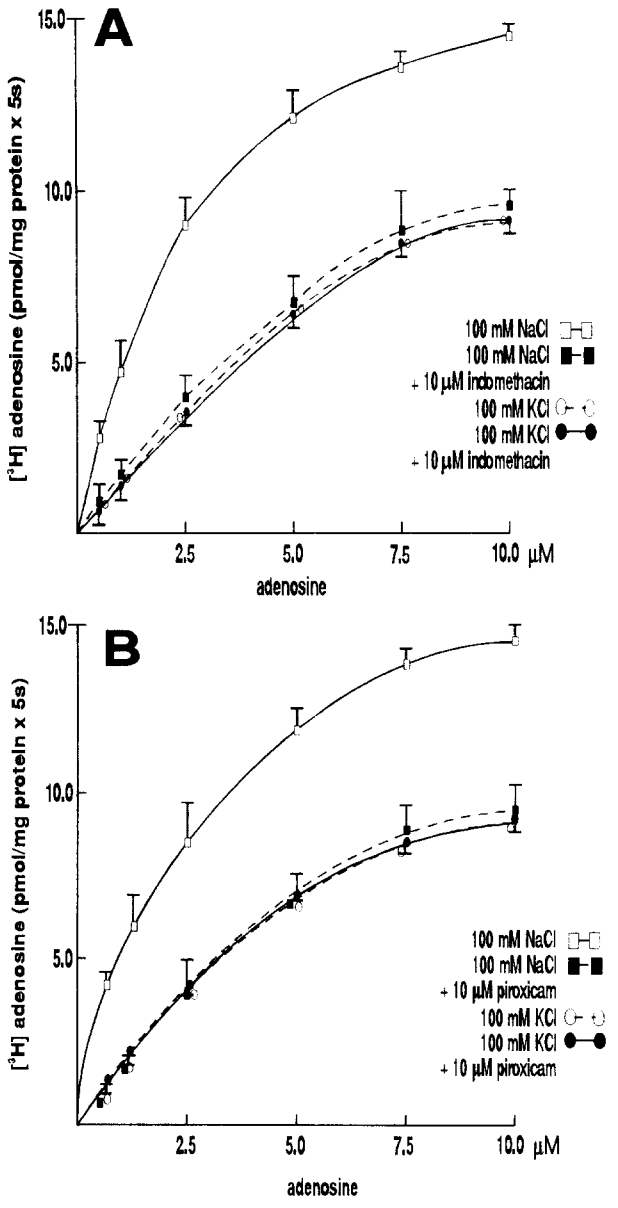

Fig. 3. Effects of maximal dose inhibition of indomethacin and piroxicam on Na^+-dependent adenosine transport in brush-border membrane vesicles. The effects of 10 μM indomethacin (A) and piroxicam (B) were evaluated on Na^+-dependent adenosine transport in the range 1–10 μM adenosine (low K_m system). The 5 s [^{3}H]adenosine transport was evaluated in the presence of 100 mM NaCl (□,■) and 100 mM KCl (○,●). Membranes were preincubated with the drugs for 60 min before transport was started ($t = 0$). Data are expressed as the means ± S.E., $n = 6$.

were 3.5 ± 0.19 and 0.1 ± 0.11 μM for indomethacin and piroxicam, respectively. The Na^+-independent adenosine transport was not affected by these drugs.

Because Na^+-dependent adenosine uptake in brush-border membrane vesicles depends on a highly specific and low K_m (2.67 ± 0.53 μM) transport system (Trimble and Coulson, 1984; Franco et al., 1990) the effect of the maximal inhibitory concentrations (5-fold the K_m value) for indomethacin (Fig. 3A) and piroxicam (Fig. 3B) were evaluated in the adenosine concentration range of 1–10 μM. In these experiments both drugs completely inhibited the Na^+-dependent adenosine transport, whereas the Na^+-independent adenosine transport kinetics were not modified.

To further determine if the blockade of adenosine transport was related to inhibition of prostanoid synthesis by the cyclooxygenase pathway, the effect of exogenous arachidonic acid on adenosine transport was evaluated. Incubation with arachidonic acid (10–50 μM) for 3 min significantly increased Na^+-dependent adenosine transport in a concentration-dependent manner (Fig. 4A). This effect was prevented by 10 μM indomethacin of all the arachidonic acid concentrations tested (Fig. 4A).

In agreement with these results, the stimulation of Na^+-dependent adenosine transport elicited by arachidonic acid showed a dose- and a time-dependent response. Effects of addition of 50 μM arachidonic acid at 1, 3 and 5 min are shown in Fig. 4B. At 1 min, arachidonic acid failed to stimulate the 5-s Na^+-adenosine transport, at 3 min a significant stimulation of Na^+-dependent adenosine transport was observed; however at 5 min the stimulation began to decrease. These effects were blocked in the presence of indomethacin.

It is likely that stimulation of Na^+-dependent adenosine transport by exogenous arachidonic acid reflects its metabolism through the cyclooxygenase pathway. Thus, prostaglandin E_2 production was determined in brush-border membranes incubated for 3 min in the presence of arachidonic acid. Basal prostaglandin E_2 production (81.77 ± 22 pg/mg protein) in the presence of 50 μM arachidonic acid was significantly stimulated (767.50 ± 123 pg/mg, $P < 0.05$ vs. basal prostaglandin E_2) and significantly inhibited when brush-border membranes were preincubated with indomethacin for 60 min (25.32 ± 9 pg/mg protein, $P < 0.05$ vs. arachidonic acid-stimulated prostaglandin E_2). Consequently the effect of exogenous prostaglandin E_2 on Na^+-dependent adenosine transport was evaluated in brush-border membranes incubated with 10 μM indomethacin. When the prostanoid (50 pM to 5 μM) was incubated for 10 min only, 5 μM prostaglandin E_2 stimulated Na^+-dependent adenosine transport 1.55 times vs. control (1.63 ± 0.34 vs. 1.05 ± 0.24 pmol/mg protein × 5 s, respectively, $P < 0.05$).

The effects of arachidonic acid metabolites synthesized through the oxidative pathways were investigated. Nordihydroguaiaretic acid, a lipoxygenase inhibitor, 17-octadecynoic acid a leukotriene-hydroxylase inhibitor and clotrimazole, a potent inhibitor of bifunctional cytochrome P_{450} monoxygenase, were preincubated for 60 min with membrane suspensions. Ten micromolar concentrations of inhibitors of 5 s Na^+-dependent adenosine transport were used. Nor-dihydroguaiaretic acid did not modify arachidonic acid stimulation (2.27 ± 0.29 nor-dihydroguaiaretic acid vs. 2.28 ± 0.28 control, vs. 3.36 ± 0.42 arachidonic acid pmol/mg protein, $P < 0.05$). Both 17-octadecynoic acid (2.11 ± 0.20 17-octadecynoic acid vs. 2.28 ± 0.28

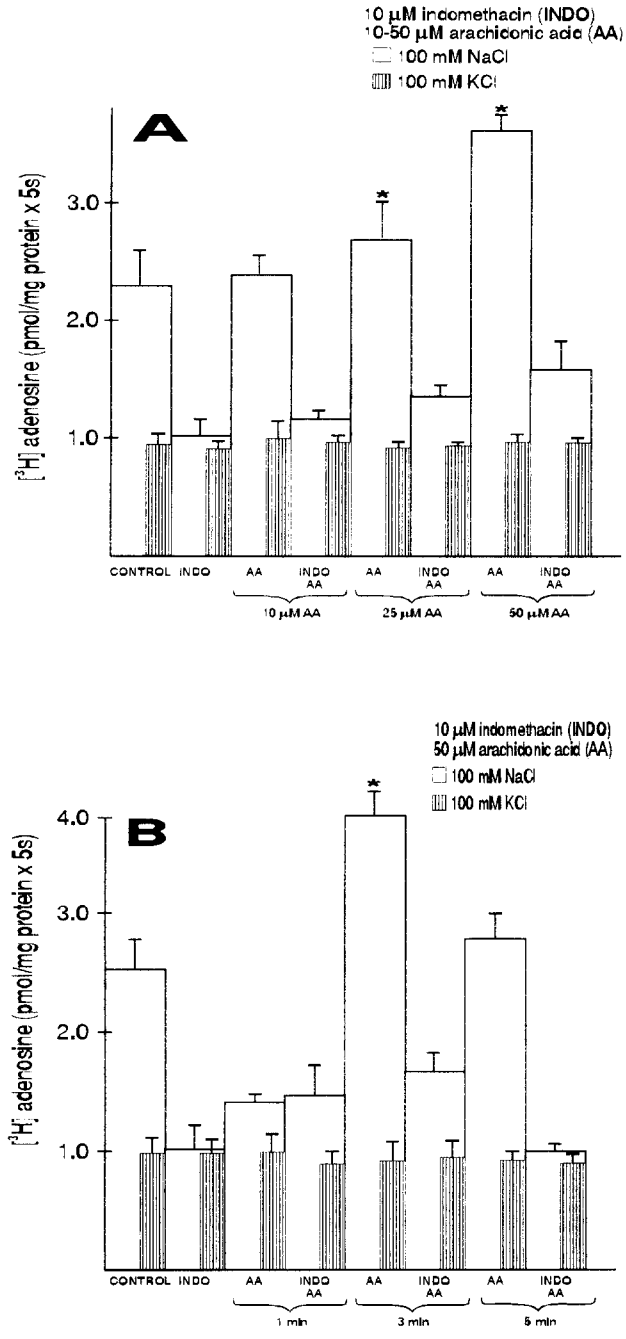

Fig. 4. Dose-dependent and time effects of arachidonic acid on Na^+-dependent adenosine transport. 1 μM [^{3}H]adenosine transport was determined at 5 s in the presence of 100 mM NaCl (open bars) and 100 mM KCl (hatched bars). Dose-dependent effects of arachidonic acid were determined at 10, 25 and 50 μM (A). Arachidonic acid was preincubated for 3 min prior to when [^{3}H]adenosine uptake was determined. Time dependence was evaluated with 50 μM arachidonic acid at various incubation times: 1, 3 and 5 min (B). Data are expressed as the means ± S.E., $n = 6$.

control, vs. 2.70 ± 0.35 arachidonic acid + 17-octadecynoic acid, pmol/mg protein) and clotrimazole (2.40 ± 0.42 clotrimazole vs. 2.28 ± 0.28 control, vs. 2.36 ± 0.36 arachidonic acid + clotrimazole, pmol/mg protein) prevented arachidonic acid stimulation, but did not inhibit Na^+-dependent adenosine transport.

4. Discussion

Our results clearly demonstrated that indomethacin and piroxicam inhibit Na^+-dependent adenosine transport in rat renal brush-border membrane vesicles. The kinetic constants K_m and V_{max}, for this transporter were similar to those reported by others (Trimble and Coulson, 1984; Franco et al., 1990). Thus the results obtained involve changes in the highly specific and low K_m system for the adenosine transporter.

Indomethacin and piroxicam inhibited the Na^+-dependent adenosine transport at 5 s. At this time adenosine transport was still linear, and purine metabolites represented less than 15%, thus we were actually measuring adenosine transport. The inhibition observed with both compounds showed time- and dose-dependent patterns with IC_{50} 3.5 μM for indomethacin and 0.1 μM for piroxicam. This inhibition was rather specific for the adenosine transporter, since Na^+-dependent lactate and Na^+-leucine transporters were not modified by either 10 μM indomethacin or piroxicam. On the other hand, Na^+-independent adenosine transport was not modified by the inhibitors. These data suggest that the cyclooxygenase-mediated inhibition could prevent the de novo synthesis of prostaglandins in these vesicles as has been demonstrated for brush-border membrane vesicles (Morduchowicz et al., 1992) and renal tissue (Hassid and Dunn, 1980; Reyes et al., 1990).

However, indomethacin effects, unrelated to cyclooxygenase inhibition, have been reported. Among them, inhibitory effects on cAMP-dependent protein kinase A, on endogenous protein phosphorylation and on phospholipase A_2 activity have been shown. These effects could be attributed to the Ca^{2+} channel antagonist properties of the drug (Franson et al., 1980). In this regard, it has been demonstrated that piroxicam, an oxicam structurally unrelated to the indoleacetic derivative, does not have as many effects as indomethacin (Carthy et al., 1980a,b), and does not interfere with calcium mobilization at concentrations below 300 μM (Burch et al., 1983). Thus, we used piroxicam to eliminate the possibility that inhibition of Na^+-dependent adenosine transport could be mediated through a non-cyclooxygenase mechanism. Piroxicam produced effects on adenosine transport similar to those described for indomethacin, supporting the hypothesis that cyclooxygenase-dependent products may participate in the regulation of Na^+-dependent adenosine transport. This possibility is further supported by the fact that the maximal inhibitory concentration of indomethacin and piroxicam (10 μM) completely abolished the low K_m high-affinity system for Na^+-adenosine transport.

Since our studies did not evaluate directly cyclooxygenase activity, it was our first priority to demonstrate a correlation between the activity of the transporter and addition of arachidonic acid. Indeed, we observed a dose-dependent response to arachidonic acid. While 10 μM arachidonic acid did not stimulate Na^+-dependent adeno-

sine transport, a significant increase was observed at 25 and 50 μM. Thus 50 μM was used for further studies. Furthermore, a time-dependent effect was also observed on Na^+-dependent adenosine transport, while the addition of arachidonic acid decreased transport at 1 min, it attained maximal stimulation at 3 min and the stimulation was less at 5 min. We can argue that the waning effect at 5 min may be due to esterification of arachidonic acid in the membranes or to metabolism. These results are in agreement with those of other studies on prostaglandin H synthase activity. These studies demonstrated that, under standard incubation conditions, the activity of the enzyme is time-dependent (Eling et al., 1990). Results of in vitro studies indicate that the effects of exogenous arachidonic acid and prostanoids last a few min in non-hormonally stimulated preparations (Badr and Brenner, 1994) and, in addition to enzymatic metabolism, these compounds exhibit spontaneous hydrolysis and oxidation (Eling et al., 1990). The participation of prostaglandins in the increased activity of the Na^+-dependent adenosine transport is further supported by the inhibition of the arachidonic acid effects by indomethacin at all arachidonic acid concentrations used and at all incubation times tested. In this regard, treatment with cyclooxygenase inhibitors abolishes the response to arachidonic acid of Na^+ reabsorption in proximal isolated tubules (Kinoshita et al., 1989), and of Na^+/K^+-ATPase activity in Madin-Darby canine kidney cells (Cohen-Luria et al., 1994).

On the other hand, it has been demonstrated that prostaglandin E_2 is synthesized upon addition of exogenous arachidonic acid in brush-border membrane vesicles (Morduchowicz et al., 1992). When we measured basal levels of prostaglandin E_2,an inhibitory effect of indomethacin (69%) was demonstrated. With the addition of arachidonic acid, a 9.4-fold increase was observed. These results suggest that prostaglandin E_2 could be related to the activity of the transporter. Since other arachidonic acid metabolites were not measured, the participation of other prostanoids cannot be ruled out. However, when prostaglandin E_2 was added to the vesicles, stimulation of the adenosine transporter was observed only at 5 μM; higher concentrations were difficult to obtain. We can argue that the concentration of prostaglandin E_2 tested would not have been sufficient to obtain a uniform distribution between the lipid-water interface in the vesicles, resulting in a concentration lower than that expected.

Arachidonic acid oxidative pathways were explored in this study through the use of the lipoxygenase inhibitors nordihydroguaiaretic acid, 17-octadecynoic acid a leukotriene-hydroxylase inhibitor and clotrimazole, a potent inhibitor of cytochrome P_{450} monoxygenase. Nordihydroguaiaretic acid did not itself modify Na^+-adenosine transport; furthermore, it did not inhibit the stimulation of adenosine transport when arachidonic acid was added, suggesting that metabolites of arachidonic acid through this pathway are not involved in the regulation of the adenosine transporter. However, in spite of the fact that 17-octadecynoic acid and clotrimazole did not modify the Na^+-adenosine transport itself, these compounds inhibited the stimulatory effect of arachidonic acid on adenosine transport. This finding suggests that oxidative products, via cytochrome P_{450}, may participate as modulators. Indeed, it has been shown than 12-, 19-, and 20-hydroxyeicosatetraenoic acids inhibit Na^+-K^+-ATPase activity and Rb^+ uptake in the thick ascending limb (Escalante et al., 1991), and Na^+-dependent phosphate and Na^+-dependent glucose transport in the proximal tubule (Friedlander et al., 1990). In this regard, epoxygenase and ω/ω-1 oxidase activities accounted for 59 ± 3 and $36 \pm 2\%$, respectively, of the total of the microsomal fraction of the cytochrome P_{450} oxidase measured in the renal cortex of the rat (Takahashi et al., 1990).

In conclusion, the activity of the Na^+-dependent adenosine transporter may be under the regulation of prostanoids; cyclooxygenase seems to be the main pathway involved; the cytochrome P_{450} and the leukotriene-hydroxylase pathways would be involved as modulators. However, the extent of the participation of these pathways will require further investigation.

Acknowledgements

This work was supported in part by grant 3007-M (CONACyT-Mexico). C. G.-C. was supported by grant 83858 (CONACyT-Mexico). We are in debt to Beatríz Vázquez and Maximina Escobedo for their technical assistance.

References

Aarab, L., Montegut, M., Siuame-Perez, S., Imbert-Teboul, M., Chabardès, C., 1993. PGE_2-induced inhibition of AVP-dependent cAMP accumulation in the OMCD of the rat kidney is cumulative with respect to the effects of α-2-adrenergic and A1-adenosine agonists, insensitive to pertussis toxin and dependent on extracellular calcium. Pflüg. Arch. 423, 397.

Badr, K.F. and Brenner, B.M., 1994, Renal prostaglandins and kinins. In: R.G. Narins (Ed.), Clinical Disorders of Fluid and Electrolyte Metabolism. McGraw-Hill, New York, NY, p. 443.

Booth, A.G., Kenny, A., 1974. A rapid method for the preparation of microvilli from rabbit kidney. J. Biochem. 145, 575.

Burch, R.M., Wise, W.C., Halushka, P.V., 1983. Prostaglandin-independent inhibition of calcium transport by nonsteroidal anti-inflamatory drugs: differential effects of carboxilic acids and piroxicam. J. Pharmacol. Exp. Ther. 227, 84.

Carthy, T.J., Eskra, J.D., Lombardino, J.G., Hoffman, W.W., 1980a. Piroxicam, a potent inhibitor of prostaglandin production in cell culture. Structure-activity study. Prostaglandins 19, 51.

Carthy, T.J., Stevens, J.S., Lombardino, J.G., Parry, M.J., Randall, M.J., 1980b. Piroxicam, a structurally novel anti-inflammatory compound. Mode of prostaglandin synthesis inhibition. Prostaglandins 19, 671.

Cohen-Luria, R., Moran, A., Rimon, G., 1994. Cyclooxygenase inhibitors suppress inhibitory effec of PGE_2 on Na^+-K^+-ATPase in MDCK cells. Am. J. Physiol. 267 (Renal Fluid Electrolyte Physiol. 36), F94.

Eling, T.E., Thompson, D.C., Foureman, G.L., Curtis, J.F., Hughes, M.F., 1990. Prostaglandin H synthase and xenobiotic oxidation. Annu. Rev. Pharmacol. Toxicol. 30, 1.

Escalante, B., Erlij, D., Falck, J.R., McGiff, J.C., 1991. Effect of cytochrome P_{450} arachidonate metabolites on ion transport in rabbit kidney loop of Henle. Science 251, 779.

Franco, R., Centelles, J.J., Kinne, R.K.H., 1990. Further characterization of adenosine transport in renal brush-border membranes. Biochim. Biophys. Acta 1024, 241.

Franson, R.C., Eisen, D., Jesse, R., Lani, C., 1980. Inhibition of highly purified mammalian PLA_2 by NSAIDs agents. Modulation by Ca^{2+} ions. Biochem. J. 186, 633.

Friedlander, G., Le Grimellec, C., Sraer, J., Amiel, C., 1990. 12-HETE modulates Na^+-coupled uptakes in proximal tubular cells: role of diacylglycerol kinase inhibition. Am. J. Physiol. 259, F816.

Gesek, F.A., Schoolwerth, A.C., 1990. Hormonal interactions with the proximal Na^+/H^+ exchanger. Am. J. Physiol. 258, F514.

Hassid, A., Dunn, M.J., 1980. Microsomal prostaglandin biosynthesis of human kidney. J. Biol. Chem. 255, 2472.

Hébert, R.L., Jacobson, H.R., Breyer, M.D., 1991. Prostaglandin E_2 inhibits sodium transport in rabbit cortical collecting duct by increasing intracellular calcium. J. Clin. Invest. 87, 1992.

Kinoshita, Y., Romero, J.C., Knox, F.G., 1989. Effect of renal interstitial infusion of arachidonic acid on proximal sodium reabsorption. Am. J. Physiol. 257, F237.

Kisaki, H., Sakurada, T., 1977. Simple micro-assay methods for enzymes of purine metabolism. J. Lab. Clin. Med. 89, 1135.

Le Hir, M., Dubach, U.C., 1984. Sodium gradient-energized concentrative transport of adenosine in renal brush border vesicles. Pflüg. Arch. 401, 58.

Martínez, F., Reyes, J.L., 1984. Prostaglandin receptors and hormonal actions on water fluxes in cultured canine renal cells (MDCK line). J. Physiol. (London) 347, 533.

Martínez, F., Manganel, M., Montrose-Rafizadeh, C., Werner, D., Roch-Ramel, F., 1990. Transport of urate and p-aminohippurate in rabbit renal brush-border membranes. Am. J. Physiol. 258, F1145.

Morduchowicz, G., Erman, A., Chen-Gal, B., Rosenfeld, J.B., Boner, G., 1992. Prostanoids and thromboxane production by renal brush border membrane. Scand. J. Clin. Lab. Invest. 52, 819.

Ouellet, M., Percival, M.D., 1995. Effect of inhibitor time-dependency on selectivity towards cyclooxigenase isoforms. Biochem. J. 306, 247.

Reyes, J.L., Meléndez, E., Martínez, F., 1990. Use of indomethacin in prostaglandin E_2 binding studies in renal cells. Pharmacodyn. Ther. 9, 153.

Siragy, H.M., Felder, R.A., Howell, N.L., Chevalier, R.L., Peach, M.J., Carey, R.M., 1989. Evidence that intrarenal dopamine acts as a paracrine substance at the renal tubule. Am. J. Physiol. 257, F469.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, I.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 85.

Smith, W.L., 1992. Prostanoid biosynthesis and mechanism of action. Am. J. Physiol. 263, F191.

Takahashi, K., Capdevila, J., Karara, A., Falck, J.R., Jacobson, H.R., Badr, K.F., 1990. Cytochrome P_{450} arachidonate metabolites in rat kidney: characterization and hemodynamic responses. Am. J. Physiol. 258, F781.

Trimble, M.E., Coulson, R., 1984. Adenosine transport in perfused rat kidney and renal cortical membrane vesicles. Am. J. Physiol. 246, F794.

Yusufi, A.K., Murayama, N., Keller, M.J., Dousa, T.P., 1985. Modulatory effect of thyroid hormones on uptake of phosphate and other solutes across luminal brush-border membrane of kidney cortex. Endocrinology 116, 2438.