# Locally formed dopamine modulates renal Na–$P_i$ co-transport through $DA_1$ and $DA_2$ receptors

Régine PERRICHOT,* Adolfo GARCIA-OCAÑA,* Sylvianne COUETTE,* Etienne COMOY,† Claude AMIEL* and Gérard FRIEDLANDER*‡

*Department of Physiology and INSERM U 426, Faculté de Médecine X.-Bichat, Université de Paris 7, Paris, France and †Department of Clinical Biology, Institut Gustave-Roussy, Villejuif, France

The involvement of dopamine (DA) receptor subtypes in regulation of renal phosphate transport by DA, either exogenous or locally synthesized from L-dihydroxyphenylalanine (L-dopa) was evaluated in opossum kidney (OK) cells with proximal tubular phenotype. DA synthesis from L-dopa by OK cells was abolished by carbidopa and benserazide, two dissimilar inhibitors of aromatic L-amino acid decarboxylase. L-Dopa stimulated cyclic AMP generation and inhibited Na-dependent $P_i$ uptake, and these effects were abolished by carbidopa and benserazide. The effects of L-dopa or DA on cyclic AMP generation and on Na–$P_i$ co-transport were mimicked by SKF 38393, a $DA_1$ receptor agonist, and were potentiated by S-sulpiride, a $DA_2$ receptor antagonist. Bromocriptine, a $DA_2$ receptor agonist, blunted in a pertussis toxin-dependent manner parathyroid hormone (PTH)-induced cyclic AMP generation and inhibition of $P_i$ uptake. In contrast with PTH, neither L-dopa nor DA affected significantly the cytosolic calcium concentration. These results support the involvement of $DA_1$ and $DA_2$ receptors, positively and negatively coupled into adenylate cyclase respectively, in modulation of renal phosphate transport.

## INTRODUCTION

Dopamine (DA) has been acknowledged as an important modulator of renal function inasmuch as it increases glomerular filtration rate and renal plasma flow and promotes natriuresis and phosphaturia (see [1] for a review). The cellular steps involved in the renal tubular effects of DA include binding to membrane DA receptors ($DA_1$ and $DA_2$), activation or inhibition of intracellular signalling pathways (adenylate cyclase–cyclic AMP–protein kinase A and phospholipase C–calcium–protein kinase C), and, finally, inhibition of transport systems responsible for sodium entry into, or exit from, the tubular cells [1–3]; the activity of Na–H exchange [4,5], Na–$P_i$ co-transport [6–10] and Na,K-ATPase [11–14] were shown to be decreased under the influence of DA. As regards $P_i$ transport, the inhibitory effect of DA was shown to be restricted to the straight proximal tubule [10,15] and to result from a primary inhibition of Na–$P_i$ co-transport activity [7,9].

Renal DA synthesis has been extensively documented and was shown to predominate in the renal cortex, mainly in proximal tubules which exhibit a high activity of aromatic L-amino acid decarboxylase (AAAD) which is the enzyme responsible for conversion of L-dihydroxyphenylalanine (L-dopa) into DA [16–21]. DA synthesis was also reported in cultured renal cells with proximal features such as LLC-$PK_1$ cells [21–23] and opossum kidney (OK) cells [24]. Tubular effects of DA were mimicked *in vivo* or *in vitro* by L-dopa, except during blockade of AAAD activity by inhibitors such as carbidopa, benserazide or hydroxybenzylhydrazine [6,7,12,14,23,24].

Recently, modulation of Na–$P_i$ co-transport by exogenous and locally synthesized DA was demonstrated in OK cells [24] in which Na–$P_i$ co-transport is inhibited during activation of protein kinases A and C [25]. However, little information is presently available concerning the DA receptor subtypes present in OK cells, their coupling to intracellular signalling pathways, and their involvement in modulation of Na–$P_i$ co-transport. The present study was undertaken to address these questions.

## MATERIALS AND METHODS

### Materials

RO 20-1724 and benserazide were gifts from Hoffman-La Roche laboratories, SKF 38393 from Smith, Kline and French, and carbidopa from Merck, Sharp and Dohme. Tracers were from the following sources: $K_2H^{32}PO_4$ from New England Nuclear (Boston, MA, U.S.A.), methyl α-D-[U-$^{14}$C]glucopyranoside ([$^{14}$C]MGP) and L-[2,3-$^3$H]alanine from Amersham (Amersham, Bucks., U.K.). Culture media and reagents were from Techgen (Les Ulis, France). Plasticware was from Costar (Cambridge, MA, U.S.A.). Other compounds were purchased from Sigma Chemical (St. Louis, MO, U.S.A.).

### Cell culture

For most experiments, OK cells (passages 80–100) were grown to confluence in 6-well or 24-well trays as previously described [26]. In some experiments, OK cells were grown on 9-mm-width glass coverslips, for determination of intracellular calcium concentration ($[Ca^{2+}]_i$), or on polycarbonate permeable supports (Transwell trays) for determination of intracellular cyclic AMP content. Monolayers reached confluence after 4 days, and they were used for experiments 2 or 3 days after confluence was achieved. On the day prior to experiments, culture medium was changed to hormone-free and serum-free medium. Cells were subcultured weekly by trypsinization. The splitting ratio was 1:5.

Abbreviations used: AAAD, aromatic L-amino acid decarboxylase; $[Ca^{2+}]_i$, intracellular calcium concentration; DA, dopamine; $DA_1$ and $DA_2$, dopamine receptors; HBS, Hanks' balanced salt solution; L-dopa, L-dihydroxyphenylalanine; MGP, methyl α-D-glucopyranoside; Na–H, sodium–proton; Na–$P_i$, sodium–phosphate; OK, opossum kidney; PTH, parathyroid hormone.

‡ To whom correspondence should be addressed.

### DA synthesis

Cells grown in 6-well trays were incubated at 37 °C in 2 ml/well of a buffered solution with the following composition (mmol/l): 137 NaCl/5.4 KCl/1 $CaCl_2$/1.2 $MgSO_4$/1 $NaHPO_4$/15 Hepes/5 glucose (pH 7.4), for 30 or 60 min. Various concentrations of L-dopa, carbidopa or benserazide were added to the medium. Stock solutions of these compounds were prepared in the same solution supplemented with 1 mM ascorbic acid in order to prevent oxidation. At the end of incubation, 1.5 ml aliquots of the incubation medium were transferred to plastic tubes containing 10 $\mu$l of 13 M HCl. Tubes were stored at 20 °C until required for analysis. Determination of DA content was performed by HPLC and electrodetection [27–29].

### Uptake studies

Uptake studies of $P_i$, MGP and alanine were performed as previously described [26,30]. Briefly, uptake experiments were performed at 37 °C in a buffered solution with the following composition (mmol/l): 137 NaCl/5.4 KCl/1 $CaCl_2$/1.2 $MgSO_4$/15 Hepes (pH 7.4). The sodium-free solution was made iso-osmotic by replacing NaCl with *N*-methyl-D-glucamine. After removal of culture medium, cells were washed with 1 ml/well of the uptake solution, and were incubated for 5 min in the presence of either $K_2H^{32}PO_4$ (0.5 $\mu$Ci/ml), [$^{14}$C]MGP (0.5 $\mu$Ci/ml) or L-[2,3-$^3$H]alanine (1 $\mu$Ci/ml) and either 100 $\mu$M $KH_2PO_4$ or 100 $\mu$M L-alanine or 1 mM MGP. All these steps were performed at 37 °C. We have shown in previous studies [30] that uptake levels increased linearly with time of incubation up to 15 min and that the Na-independent component of uptake represented less than 10% of total uptake for phosphate and glucose, and less than 20% of total uptake for alanine. At the end of incubation, the uptake was stopped by washing the cells three times with 1 ml/well of ice-cold solution (137 mM NaCl/15 mM Hepes, pH 7.4). Cells were then solubilized in 0.5% Triton X-100 (250 $\mu$l/well) and aliquots were counted by liquid scintillation.

### Determination of cyclic AMP content

After removal of culture medium, cells were washed with 1 ml/well of Hanks' balanced salt solution (HBS) supplemented with 15 mM Hepes and 2 mM L-glutamine (HBS-Hepes), and were then preincubated for 15 min at 37 °C in HBS-Hepes at pH 7.4 (500 $\mu$l/well) containing 1 mM ascorbic acid and RO 20-1724, a specific inhibitor of type-IV phosphodiesterase, which was used at a concentration of 100 $\mu$M. Medium was then removed and cells were incubated, usually for 20 min, in a similar solution to which hormones were added. At the end of the incubation period, the intracellular cyclic AMP content was measured by radioimmunoassay as previously described [31]. In some experiments, cells grown on permeable supports were incubated under similar conditions with hormones added either to the apical or to the basal medium.

### $[Ca^{2+}]_i$ measurement

Confluent cells were loaded at 37 °C for 10 min with 1.5 $\mu$M Fura 2-AM dissolved in culture medium. After trypsinization (0.05% trypsin/0.02% EDTA, 3 min, 37 °C), cells were washed twice with 10 ml of PBS and the final pellet was resuspended in 6 ml of PBS. A 2 ml aliquot of cell suspension was transferred in a quartz cuvette and fluorescence measurements were monitored at 37 °C, under constant stirring, in a F2000 Hitachi spectrofluorimeter at 340 nm, 370 nm (excitation wavelengths) and 540 nm (emission wavelength). Maximal fluorescence was obtained with 50 $\mu$M digitonin and minimal fluorescence with 10 mM EGTA. $[Ca^{2+}]_i$ was calculated with a $K_d$ value for Fura 2 equal to 224 nM. The values of $[Ca^{2+}]_i$ given in the results were those determined at the peak of intracellular calcium increase as described previously [26].

In a separate set of experiments, $[Ca^{2+}]_i$ was determined on attached cells grown on glass coverslips. Cells were loaded at 37 °C for 15 min with 1.5 $\mu$M Fura 2-AM and coverslips were inserted in the cuvette and held at a fixed angle. $[Ca^{2+}]_i$ was determined as above.

### Presentation of data

Uptake levels were expressed as nmol/mg of protein. Protein contents were measured according to Bradford with BSA as standard [32]. Albumin was diluted in Triton in order to treat standard samples in the same way as experimental ones. DA accumulation in the medium was expressed as pmol/ml and intracellular cyclic AMP content was expressed as pmol/mg of protein. Na-dependent uptake levels were calculated by subtracting uptake values measured in the presence of *N*-methyl-D-glucamine from those measured in the presence of Na. Results were presented as means ± S.E.M. of three to five different experiments ($n$) in which duplicates or triplicates were obtained. One-way or two-way analyses of variance were performed and, when allowed by the $F$ value, results were compared by the modified $t$-test.

## RESULTS

### DA synthesis by OK cells

In a preliminary set of experiments, we verified that, in our model, OK cells converted L-dopa into DA. As shown in Figure 1, DA accumulation in the medium was time- and substrate-dependent. Carbidopa and benserazide reduced DA accumulation dramatically (Table 1).

### Effect of DA and L-dopa on cyclic AMP generation

In another preliminary set of experiments, we verified that, in our hands, DA receptors, positively coupled to adenylate cyclase, were expressed in OK cells. Indeed, intracellular cyclic AMP

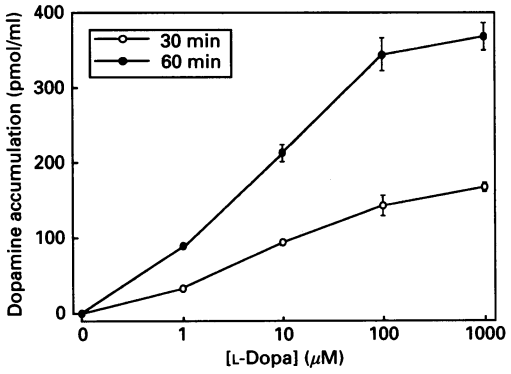


**Figure 1 DA synthesis by OK cells**

OK cells were incubated with L-dopa at the indicated concentrations for 30 or 60 min. At the end of incubation, supernatants were acidified and stored at 20 °C until measurement of DA was performed as indicated in the Materials and methods section. Results are presented as means ± S.E.M. of four different experiments ($n$ = 4) in which duplicates were obtained.

**Table 1 Effect of carbidopa and benserazide on DA synthesis by OK cells**

OK cells were incubated for 30 min or 60 min with $10^{-4}$ M L-dopa in the presence or absence of carbidopa or benserazide, each of them at $10^{-4}$ M. Results are presented as means ± S.E.M. of four different experiments ($n = 4$) in which duplicates were obtained.

| | Dopamine accumulation (pmol/ml) | | |
|---|---|---|---|
| Incubation | Control | Carbidopa | Benserazide |
| 30 min | 143 ± 14.0 | 3 ± 0.2 | 12 ± 3.4 |
| 60 min | 343 ± 22.1 | 5 ± 1.9 | 23 ± 6.7 |

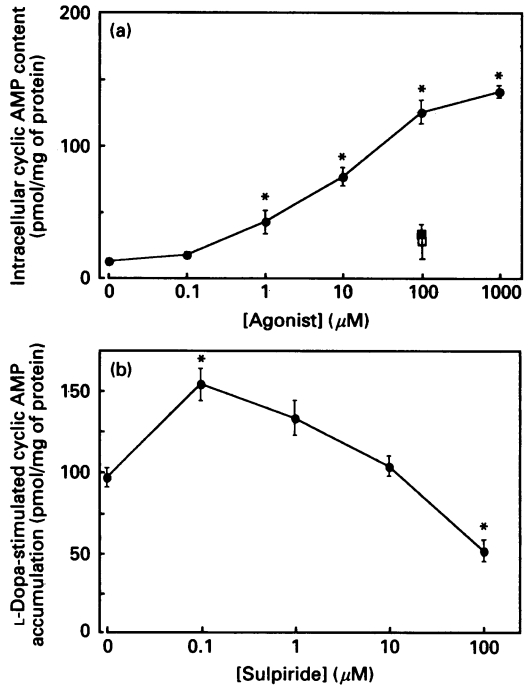


**Figure 2 Effect of L-dopa on intracellular cyclic AMP content in OK cells**

(**a**) OK cells were incubated for 20 min in the presence of L-dopa at the indicated concentrations. Benserazide (□) or carbidopa (■) were added at a concentration of 100 μM. *Significantly different from the basal value without agonist ($P < 0.05$). (**b**) OK cells were incubated for 20 min with L-dopa (100 μM) and increasing concentrations of sulpiride. * Significantly different from the value without sulpiride ($P < 0.05$). Results are expressed as means ± S.E.M. of four different experiments ($n = 4$) in which duplicates were obtained.

content increased from the unstimulated value of 13 ± 1.1 pmol/mg of protein to 21.5 ± 3.0, 74 ± 20.2 and 142 ± 31.5 pmol/mg of protein after incubation with DA at concentrations of 0.1, 1 and 10 μM respectively. L-Dopa increased intracellular cyclic AMP content in a concentration-dependent manner. The threshold was 0.1 μM and a plateau for stimulation was reached at 100 μM (Figure 2a). Benserazide and carbidopa blocked the effect of L-dopa but did not affect the stimulation induced by DA (results not shown).

The $DA_2$ receptor agonist bromocriptine reduced parathyroid hormone (PTH)- and L-dopa-induced cyclic AMP accumulation significantly. Pretreatment of the cells with pertussis toxin, which

**Table 2 Effect of bromocriptine and pertussis toxin on cyclic AMP accumulation in OK cells**

OK cells were incubated for 20 min with or without PTH (10 nM) or L-dopa (100 μM) in the absence or presence of bromocriptine (100 nM). Pretreatment with pertussis toxin (PTX; 250 ng/ml) was performed for 18 h prior to experiments.

| | Intracellular cyclic AMP content (pmol/mg of protein) | |
|---|---|---|
| | Control | Bromocriptine |
| Basal | 21 ± 6 | 14 ± 5 |
| PTH | 1191 ± 95 | 636 ± 85* |
| PTH + PTX | 1218 ± 130 | 1329 ± 125† |
| L-Dopa | 194 ± 9 | 123 ± 12* |
| L-Dopa + PTX | 370 ± 21† | 385 ± 26† |

* Significantly different from the corresponding value without bromocriptine ($P < 0.05$)
† Significantly different from the corresponding value without pertussis toxin ($P < 0.05$).

**Table 3 Effect of L-dopa, DA and PTH on $[Ca^{2+}]_i$ in OK cells**

$[Ca^{2+}]_i$ was measured in OK cells under basal conditions and after addition of DA, L-dopa, or PTH at the indicated concentrations. Results are expressed as means ± S.E.M. of four different experiments ($n = 4$) in which triplicates were obtained. Abbreviation: ND, not determined.

| | $[Ca^{2+}]_i$ (nM) | | | |
|---|---|---|---|---|
| | Suspended cells | | Attached cells | |
| | Control | Experimental | Control | Experimental |
| Dopamine ($10^{-5}$ M) | 155 ± 25 | 147 ± 18 | 112 ± 20 | 134 ± 27 |
| L-Dopa ($10^{-5}$ M) | 146 ± 14 | 152 ± 12 | ND | ND |
| PTH ($10^{-7}$ M) | 143 ± 12 | 239 ± 22* | 120 ± 18 | 312 ± 22* |

* Significantly different from the control condition ($P < 0.05$).

inactivates $G_i$ protein through its ADP-ribosylation, abolished the effect of bromocriptine and enhanced the stimulatory effect of L-dopa on cyclic AMP generation (Table 2).

S-sulpiride, which is a selective $DA_2$ receptor antagonist at low concentrations (0.1 μM), potentiated the stimulatory effect of L-dopa (Figure 2b). At high concentrations (100 μM), S-sulpiride inhibited the effect of L-dopa.

SKF 38393, a $DA_1$ agonist, at 1 μM mimicked the effect of DA on cyclic AMP accumulation (22 ± 6 to 125 ± 23 pmol/mg of protein, $P < 0.01$).

Finally, in order to characterize the sidedness of DA receptors, we measured cyclic AMP generation in OK cells grown on permeable supports. Intracellular cyclic AMP content (pmol/mg of protein) increased from 35 ± 5 to 180 ± 25 and 154 ± 12 after addition of 100 μM L-dopa to the apical or to the basal medium respectively ($P < 0.01$ versus control for both values). In the presence of 100 μM L-dopa in the basal medium, 100 nM bromocriptine applied to the apical or the basal medium decreased cyclic AMP content from 154 ± 12 to 111 ± 12 and 97 ± 10 pmol/mg of protein respectively ($P < 0.05$ versus value with L-dopa alone).

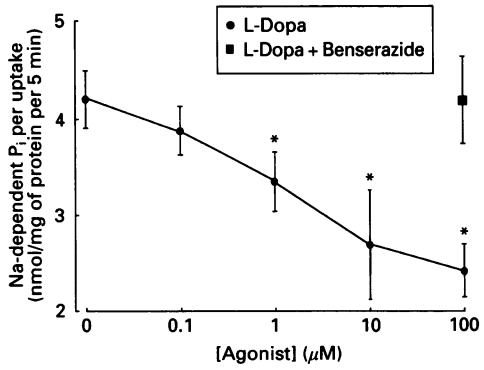


**Figure 3 Effect of L-dopa on Na-dependent $P_i$ uptake in OK cells**

OK cells were preincubated for 30 min with the indicated concentrations of L-dopa prior to measurement of $P_i$ uptake. Benserazide was added to the preincubation medium in the presence of L-dopa (■) at a concentration of 100 μM. Results are expressed as means ± S.E.M. of four different experiments ($n = 4$) in which duplicates were obtained. *Significantly different from the value without agonist ($P < 0.05$).

**Table 4 Effect of sulpiride on Na–$P_i$ co-transport inhibition by L-dopa in OK cells**

OK cells were incubated in the absence or presence of $10^{-5}$ M L-dopa with the indicated concentrations of S-sulpiride for 30 min prior to $P_i$ uptake.

| | Na-dependent $P_i$ uptake (nmol/mg of protein per 5 min) | |
|---|---|---|
| Sulpiride | Control | L-Dopa |
| 0 | 4.5 ± 0.32 | 2.8 ± 0.25* |
| $10^{-7}$ M | 4.7 ± 0.30 | 1.9 ± 0.20*† |
| $10^{-4}$ M | 4.5 ± 0.25 | 3.6 ± 0.12† |

* Significantly different from the corresponding value without L-dopa ($P < 0.05$); † significantly different from the corresponding value without sulpiride ($P < 0.05$).

### Effect of DA and L-dopa on $[Ca^{2+}]_i$

DA and L-dopa, each of them at 10 μM, had no influence on $[Ca^{2+}]_i$ (Table 3) while 100 nM PTH increased $[Ca^{2+}]_i$ significantly. Similar results were obtained whether $[Ca^{2+}]_i$ was determined in suspended or attached cells.

### Effect of DA and L-dopa on Na-dependent uptake levels

Na-dependent $P_i$ uptake was decreased from the basal value of 4.2 ± 0.3 nmol/mg of protein per 5 min to 3.3 ± 0.5, 2.8 ± 0.3 and 2.7 ± 0.2 nmol/mg of protein per 5 min when cells were preincubated for 30 min with DA at concentrations of 0.1, 1 and 10 μM respectively ($P < 0.05$ for the value with 1 or 10 μM DA versus control). L-Dopa inhibited Na-dependent $P_i$ uptake to a similar extent (Figure 3). SKF 38393 inhibited Na–$P_i$ co-transport from 3.9 ± 0.35 to 2.3 ± 0.15 nmol/mg of protein per 5 min ($n = 4$, $P < 0.01$). Benserazide, as was the case for cyclic AMP generation, abolished the effect of L-dopa (Figure 3).

The effect of DA, either added to the medium or synthesized by OK cells from L-dopa, was selective since 100 μM of DA or of L-dopa did not affect Na-dependent alanine uptake (10.5 ± 1.4, 12.0 ± 0.9, or 10.0 ± 1.5 nmol/mg of protein per 5 min with vehicle, DA and L-dopa respectively; not significant) or MGP uptake (4.0 ± 0.5, 4.2 ± 0.3, 3.6 ± 0.1 nmol/mg of protein per 5 min with vehicle, DA and L-dopa, respectively; not significant).

S-sulpiride, at a low concentration, magnified the inhibitory effect of DA on $P_i$ uptake (Table 4). At 100 μM, sulpiride blunted the effect of DA so that this effect was no longer significant. At any concentration tested sulpiride alone did not modify $P_i$ uptake (Table 4). Finally, bromocriptine, at $10^{-7}$ M, reduced the inhibition of $P_i$ uptake induced by $10^{-8}$ M PTH from 31.5 ± 4.0% to 8.5 ± 3.4% ($P < 0.005$).

## DISCUSSION

The main results of this study are that: (i) locally formed DA modulates Na–$P_i$ co-transport in OK cells; (ii) both $DA_1$ and $DA_2$ receptors, positively and negatively coupled to adenylate cyclase respectively, are involved in this response.

That locally formed DA regulated Na–$P_i$ co-transport can be deduced from the following: (i) OK cells synthesized DA from L-dopa in a time- and concentration-dependent manner, a feature that these cells share with proximal tubular cells from dog and rat [6,21], and with porcine LLC-$PK_1$ cells [22]; (ii) L-dopa reproduced the inhibitory effect of DA on $P_i$ uptake, a result in line with those obtained by Glahn et al. *in vitro* [24] and by several groups in *in vivo* studies on various animal species [6,7]; (iii) carbidopa and benserazide, two well documented inhibitors of AAAD that blocked DA synthesis in OK cells ([24] and Table 1), prevented the inhibition of $P_i$ uptake by L-dopa.

The effect of L-dopa and DA on $P_i$ transport is most likely a primary effect on the activity of the Na–$P_i$ co-transport system rather than a consequence of dissipation of the transmembrane sodium gradient, inasmuch as neither L-dopa nor DA affected Na-coupled uptake of alanine and MGP. Although inhibition of Na,K-ATPase by endogenous DA in proximal tubular cells has been repeatedly reported [11–14], inhibition of co-transport systems inserted in proximal brush-border membranes, such as Na–$P_i$ co-transport or Na–H exchange, were shown to occur independently from the effect on the sodium pump [5,9].

As regards DA receptor subtypes which transduced the effects of DA, our results provide the first evidence that both $DA_1$ and $DA_2$ receptors are involved in modulation of $P_i$ transport. Involvement of $DA_1$ receptors results from the following: (i) both L-dopa and DA raised intracellular cyclic AMP content, a feature consistent with the presence of receptors which are positively linked to adenylate cyclase in OK cells, as reported by several groups [33,34]; (ii) SKF 38393, a $DA_1$ receptor agonist [1,3,35], reproduced the effect of DA on cyclic AMP content and $P_i$ uptake. Concerning $DA_2$ receptors, negatively coupled to adenylate cyclase [3,36], their presence in OK cells is attested by several features: (i) S-sulpiride, a selective $DA_2$ antagonist at low concentrations [35], enhanced cyclic AMP generation elicited by L-dopa, whereas bromocriptine, a $DA_2$ agonist [35], decreased PTH- and L-dopa-induced cyclic AMP synthesis; (ii) inactivation of $G_i$ protein by pertussis toxin magnified L-dopa-induced cyclic AMP generation and blunted the inhibitory effect of bromocriptine. More importantly, modulation of Na–$P_i$ co-transport by DA involved $DA_2$ receptors since sulpiride, at 0.1 μM, enhanced the inhibitory effect of L-dopa and, in contrast, bromocriptine blunted the inhibition of $P_i$ transport by PTH. These results are in line with those of pharmacological [37–39] and molecular biological studies which have established the presence of receptors belonging to the $D_1$ and $D_2$ families in renal proximal tubular cells [40,41]. In our model, results of experiments with cells grown on permeable supports suggest that both

$DA_1$ and $DA_2$ receptors are expressed in the apical and basolateral domains of the plasma membrane.

That S-sulpiride magnified the response of OK cells to L-dopa, both in terms of cyclic AMP accumulation and of inhibition of $P_i$ transport, indicates that $DA_1$ and $DA_2$ receptors exert opposite effects on the cascade of events which leads to modulation of $P_i$ uptake. This pattern differs from that reported in modulation of Na,K-ATPase activity in proximal tubular cells: inhibition of the pump was shown to require occupancy of both receptor subtypes which act synergistically [13]. This suggests that another transduction pathway, distinct from cyclic AMP–protein kinase A activation, was involved. Along the same line, modulation by DA of Na–H exchange activity in brush-border membranes was reported to occur via cyclic AMP-dependent and cyclic AMP-independent mechanisms [4,5]. Since we and others have documented that activation of the phospholipase C–protein kinase C pathway leads to inhibition of $P_i$ uptake [25,30], we evaluated whether DA was able to increase the cytosolic calcium concentration in OK cells. That DA, in contrast with PTH, did not elicit any calcium transient in suspended or in attached cells ([26] and Table 3) argues against the involvement of this pathway in modulation of Na–$P_i$ co-transport by DA.

In summary, this work provides evidence that $DA_1$ and $DA_2$ receptors are instrumental in modulation of renal phosphate transport by locally formed DA. Whether abnormal expression of one of these receptor subtypes leads to impaired renal phosphate handling remains to be established. However, this pattern may provide clues for therapeutic approaches of hypophosphataemia related to renal phosphate leak.

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