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STRUCTURAL REQUIREMENT OF MONOPHOSPHATES FOR INHIBITION OF Na^+-P_i COTRANSPORT IN RENAL BRUSH BORDER MEMBRANE

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Abstract—Using the chemical structural analogs of phosphonoacetic acid (PAA) and related phosphonate compounds, we investigated which structural features are required for competitive inhibition of Na^+-P_i cotransport in rat renal cortical brush border membrane (BBM) vesicles (BBMV). The effects of compounds on [$Na_0^+ > Na_i^+$]-gradient-dependent $^{32}P_i$ uptake by BBMV were examined using various inhibitor-to-$^{32}P_i$ concentration ratios in the transport assay medium. The replacement of a phosphono-group with an arsono-group in PAA, or the substitution of a carboxylic group in PAA by an amino or hydroxyl group, totally abolished the inhibitory action on Na^+-P_i cotransport. Decreased electronegativity of carboxyl in PAA by coupling with hydrazine or hydroxylamine lowered the inhibitory potency of PAA. Substitution of H at the α-carbon of PAA with ethyl or *p*-Cl-phenyl groups completely abolished the inhibitory activity, whereas α-halogenation with Br greatly increased the inhibitory potency of PAA, close to that of phosphonoformic acid (PFA). The inhibition by all the active tested monophosphates was strictly competitive. The tested compounds displaced [^{14}C]PFA pre-bound onto BBMV in the presence of 100 mM NaCl. The ability of monophosphates to inhibit Na^+-P_i cotransport across BBM and the binding of [^{14}C]PFA were closely correlated ($r = 0.925$; $P < 0.001$). These results show that: (a) strong electronegativity at both ends of the PAA molecule is needed for inhibitory action, (b) an α-aliphatic or aromatic substituent at the α-carbon probably hinders the acess of the inhibitor to the P_i-binding site of the Na^+-P_i cotransporter in BBM, whereas (c) an α-electrophilic substituent—Br—enhances the inhibitory potency of PAA. The tested compounds inhibited Na^+-P_i cotransport by binding, in the presence of Na^+, on the same site on the luminal surface of BBM as did PFA and, by extension, P_i.

We reported recently that phosphonoformic acid (PFA) and phosphonoacetic acid (PAA) act as specific competitive inhibitors of Na^+-gradient-dependent [$Na_0^+ > Na_i^+$] transport of P_i across brush border membrane (BBM) in renal and intestinal epithelia [1–3]. Furthermore, studies with [^{14}C]PFA used as a ligand indicated that this compound combines with Na^+ cations and binds at the luminal face of BBM of renal proximal tubules, most likely at the locus identical with the binding sites for phosphate (P_i) of the Na^+-P_i cotransporter [4–6].

To further determine the structural requirement of phosphonocarboxylic acids and related monophosphonates as inhibitors and probes for interaction of P_i and Na^+ with the Na^+-P_i cotransporter and to examine the structural properties of the exofacial moiety of the Na^+-P_i cotransporter in BBM of renal proximal tubules [7], we studied the effects of some congeners of PAA modified at several key sites of the molecule [8, 9] and some related monophosphonates for their abilities to interact with the Na^+-P_i cotransporter in BBM vesicles (BBMV) isolated from rat renal cortex. Our results indicate that alkylphosphonates with the negatively-charged moiety of the molecule opposite to phosphonyl groups, such as the carboxylic group, and without a bulky substituent on the α-carbon, are the features required for the inhibitory effect on Na^+-P_i cotransport across BBM as well as binding on the Na^+-P_i cotransporter.

MATERIALS AND METHODS

The BBM vesicles were prepared from renal cortex of adult male Sprague–Dawley rats with use of the Mg^{2+}-precipitation method, as described in our previous papers [1, 3, 4, 6, 10, 11]. The measurement of $^{32}P_i$ uptake by BBMV and the determination of the [^{14}C]PFA binding on BBMV were conducted with use of a rapid filtration technique, as described in detail in previous reports [4–5]. In the $^{32}P_i$ uptake assays the final concentration of $^{32}P_i$ was 0.1 mM, unless indicated otherwise; in the binding assays [4], the final concentration of [^{14}C]PFA as a ligand was 0.8 mM. All tested compounds were dissolved in a medium containing 300 mM mannitol and buffered with 5 mM Tris-HEPES†, pH 7.5 (referred to as the "MTH medium" [1, 4]). This medium and other solutions were always kept at osmolality ≅ 300 mOsM. Both $^{32}P_i$ uptake by BBMV and the [^{14}C]PFA binding on BBMV were determined at 20°, at time periods indicated in the Results, in the presence of inwardly directed sodium gradient [Na^+ extravesicular > Na^+ intravesicular; $Na_0^+ > Na_i^+$] with $Na_0^+ = 100$ mM and $Na_i^+ = 0$. The Na^+-independent component [1, 6, 12] of $^{32}P_i$ uptake by BBMV in the initial (5 or 10 sec) uptake phases

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† HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

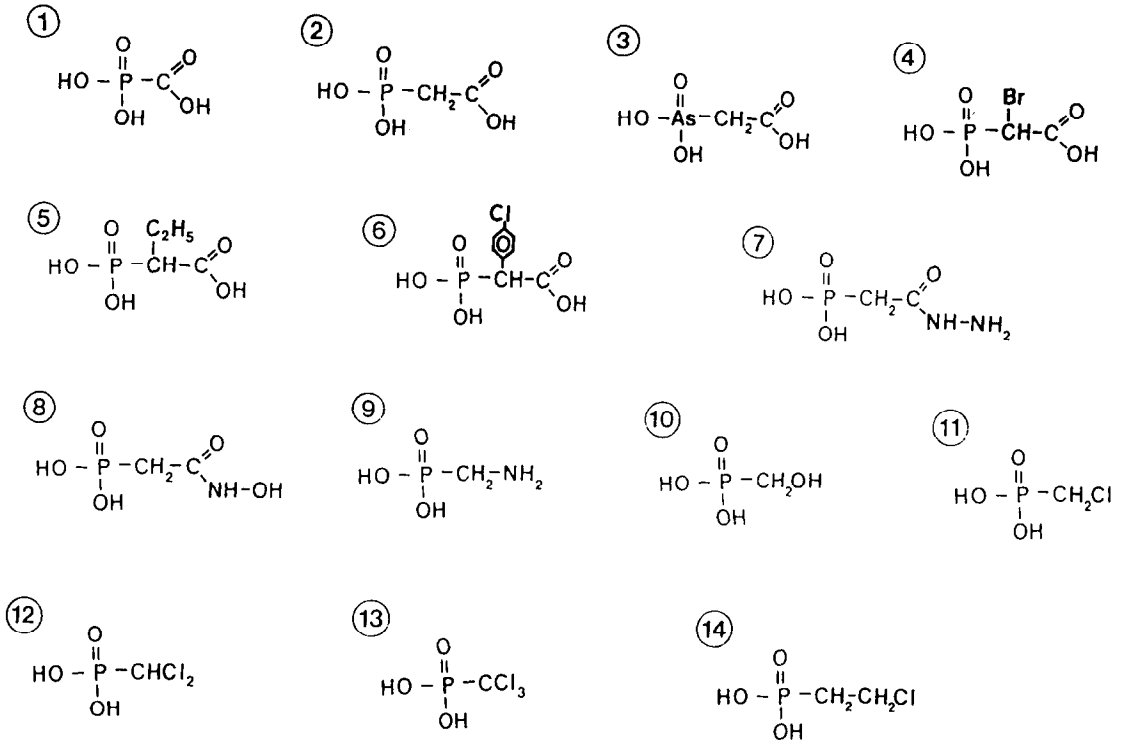

Fig. 1. Structural formulas of compounds examined in this study. In Figs 2 and 3, the compounds are denoted by the assigned numbers. Key: (1) phosphonoformic acid (PFA); (2) phosphonoacetic acid (PAA); (3) arsonoacetic acid (AsAA); (4) α-bromo-phosphonoacetic acid (α-Br-PAA); (5) α-ethyl-phosphonoacetic acid (α-ethyl-PAA); (6) α-p-chloro-phenyl-phosphonoacetic acid; (7) hydrazidophosphonoacetic acid (hydrazido-PAA); (8) hydroxylamidophosphonoacetic acid (hydroxyl-amido-PAA); (9) aminoethylphosphonic acid; (10) hydroxymethylphosphonic acid; (11) mono-chloromethylphosphonic acid; (12) dichloromethylphosphonic acid; (13) trichloromethylphosphonic acid; and (14) β-chloro-ethyl-phosphonic acid.

is negligible (≈5%); therefore, this value was not routinely measured and subtracted in the present experiments, as in our preceding studies [2, 4–6, 10, 11]. The protein content was determined by the method of Lowry *et al.* [13] as in our previous studies [1, 4, 6, 10].

The compounds examined in the present study (Fig. 1) were tested for content of P_i and stability upon incubation with BBM, similarly as in our previous study [1]. None of the compounds (Fig. 1), at least in concentrations employed for testing for the inhibitory activity, contained detectable P_i. Likewise, after a 20-min incubation of the tested compounds with BBM, under the conditions employed for [^{14}C]PFA binding experiments (see Results, Table 3), no detectable release of P_i was found.

Each experiment was conducted at least twice or more times if appropriate, and the results were evaluated statistically with use of Student's *t*-tests for group or paired comparisons.

Materials. [^{14}C]Phosphonoformic acid trisodium salt, [^{14}C]PFA, was custom-synthesized and purchased from the Amersham Co. (Arlington Heights, IL). The reported radiochemical purity of [^{14}C]PFA (by thin-layer chromotography) was 98–99%, and the specific radioactivity was 8.5 mCi/mmol (315 MBq/mmol). The [^{14}C]PFA was stored divided in aliquots at −80°, and solutions of desired specific radioactivity were prepared by admixture of unlabeled PFA, prior to the experiments. The other radiolabeled compounds, namely $^{32}P_i$, were purchased from New England Nuclear (Boston, MA).

PFA (trisodium salt hexahydrate), phosphonoacetic acid (PAA), phosphonopropionic acid (PPA), other alkylphosphonic acids (Fig. 1) and other reagents and biochemicals [1], all of the highest purity grades, were purchased from standard suppliers. Derivatives of PAA (Fig. 1): hydrazido-PAA, hydroxylamido-PAA; α-Br-PAA, α-ethyl-PAA, α-pCl-phenyl-PAA, arsonacetic acid (AsAA) and some other monophosphate derivatives including aminomethylphosphonic acid, trichloromethylphosphonic acid, monochloromethylphosphonic acid and β-Cl-ethyl-phosphonic acid were all gifts of James C. Mao, Ph.D., Senior Biochemist, Anti-Infection Drugs Section, Pharmaceutical Discovery Division, Abbot Laboratories, North Chicago, IL. Dichloromethylphosphonic acid, hydroxymethylphosphonic acid, and monochloromethylphosphonic acid were purchased from either the Sigma Chemical Co. (St Louis, MO) or the Alpha Corp., Inc.

RESULTS

The effects of the studied monophosphates, added to a final concentration of 2 mM, on the Na^+-gradient-[$Na_o^+ > Na_i^+$]-dependent $^{32}P_i$ uptake in the

Table 1. Effects of phosphonoacetic acid and related compounds on $^{32}P_i$ uptake by BBMV in the presence of [$Na_o^+ > Na_i^+$]-gradient

Additions	N	(A) P_i uptake with 0.1 mM $^{32}P_i$ in the medium (20:1 ratio) Uphill uptake (10 sec)	$-\Delta\%$*	Equilibrium uptake (120 min)	N	(B) P_i uptake with 5 μM $^{32}P_i$ in the medium (400:1 ratio) Uphill uptake (10 sec)	$-\Delta\%$*
None (control)	(3)	742 ± 50†		244 ± 29	(3)	108 ± 10	
Compound							
1 Phosphonoformic acid (PFA)	(3)	298 ± 27	−58 ± 3	218 ± 15	(3)	28 ± 3	−74 ± 5
2 Phosphonoacetic acid (PAA)	(3)	623 ± 44	−18 ± 1	215 ± 11	(3)	60 ± 6	−44 ± 3
3 Arsonoacetic acid (AsAA)	(3)	749 ± 54	ND	230 ± 22	(3)	103 ± 9	ND
4 δ-Bromo-phosphonoacetic acid (α-Br-PAA)	(3)	340 ± 16	−54 ± 3	256 ± 32	(3)	31 ± 3	−71 ± 5
5 α-Ethyl-phosphonoacetic acid α-p-Cl-Phenyl-phosphonoacetic acid (α-EtOH-PAA)	(3)	738 ± 57	ND	220 ± 28	(3)	106 ± 11	ND
6 α-p-Cl-Phenyl-phosphonoacetic acid (α-pCl-Phe-PAA)	(3)	742 ± 53	ND	239 ± 27	(2)	99 ± 5	ND
7 Hydrazidophosphonoacetic acid	(3)	635 ± 37	−14 ± 2	242 ± 33	(3)	71 ± 6	−34 ± 3
8 Hydroxylamidophosphonoacetic acid	(3)	652 ± 40	−12 ± 2	207 ± 19	(3)	73 ± 7	−32 ± 5
9 Aminomethylphosphonic acid	(3)	740 ± 46	ND	214 ± 21	(3)	115 ± 15	ND
10 Hydroxymethylphosphonic acid	(3)	746 ± 66	ND	210 ± 19	(3)	123 ± 10	ND
11 Monochloromethylphosphonic acid	(3)	652 ± 40	−13 ± 2	234 ± 12	(3)	74 ± 5	−34 ± 2
12 Dichloromethylphosphonic acid	(3)	745 ± 54	ND	229 ± 23	(2)	105 ± 3	ND
13 Trichloromethylphosphonic acid	(3)	731 ± 69	ND	235 ± 35	(2)	118 ± 4	ND
14 β-Chloro-ethyl-phosphonic acid	(3)	482 ± 19	−35 ± 3	218 ± 41	(3)	52 ± 4	−52 ± 3

In all experiments the concentration of the added test compounds was 2 mM.

* Relative (percent) inhibition compared to control (no test compounds added); all values were significant ($P < 0.05$, or higher degree of significance; *t*-test). ND = no detectable inhibition (less than 3% difference from the controls).

† Denotes $^{32}P_i$ uptake in pmol $^{32}P_i$/mg protein of BBMV per indicated incubation time; each value is the mean ± SE of experiments N (in parentheses).

uphill time phase (10 sec) were examined (Table 1) at two concentrations of $^{32}P_i$, 100 or 5 μM, that is, using inhibitor-to-solute ratios 20:1 (Table 1A) and 400:1 (Table 1B). The tested compounds were added to the assay mixture immediately before measurement of $^{32}P_i$ uptake by BBMV. The results (Table 1) show that besides PFA and PAA, from the other examined phosphonocarboxylate compounds, hydrazido-PAA, hydroxylamino-PAA and, in particular α-Br-PAA, exhibits a distinct inhibitory effect. The most potent inhibitor, α-Br-PAA, was examined for solute-specificity of the inhibition. As in the case of other previously studied phosphonocarboxylates [1, 14], α-Br-PAA had no inhibitory effect on Na^+-gradient-dependent uptake of D-glucose, L-proline, succinate or citrate by BBMV (Szczepanska-Konkel M and Dousa TP, unpublished observations). From monophosphonates without a carboxylic group in the molecule, β-chloro-ethyl-phosphonic acid and, to a lesser degree, monochloromethylphosphonic acid showed an inhibitory effect (Table 1). Invariably, the inhibition was more pronounced at 400:1 ratio (Table 1B) than at a 20:1 ratio (Table 1A). Other tested compounds, at least up to a concentration of 2 mM, neither decreased nor increased the P_i uptake by BBMV, even at a high (400:1) compound-to-phosphonate ratio (Table 1B). None of the tested compounds significantly influenced the equilibrium uptake (120 min) of P_i (Table 1A).

Next, the kinetic nature of the inhibitory effect of those compounds which turned out to be effective in the initial experiments (Table 1) was examined. The initial (5 sec) Na^+-gradient-dependent uptake of $^{32}P_i$ was measured at $^{32}P_i$ concentrations of 10, 25, 50, 75, and 100 μM in the presence of a 1 or 2 mM concentration of the test compound, and the results were evaluated by Lineweaver–Burk double-reciprocal plots (Fig. 2, Table 2). All inhibitory effects observed were strictly competitive; none of the compounds showed a statistically significant effect on V_{max} (Table 2). Thus, the efficacy of various phosphonate compounds differs only in their affinity towards the Na^+-P_i cotransporter (Table 2, Fig. 2).

Since the preceding experiments revealed a competitive type of inhibitory effects, we examined the abilities of these compounds to compete with [^{14}C]PFA for binding onto BBMV in the presence of 100 mM NaCl [4, 6]. Freshly prepared BBMV were preincubated with 0.8 mM [^{14}C]PFA in a medium containing 100 mM NaCl for 20 min; then tested compounds were added in a final concentration of 2 or 15 mM and incubation was continued for the next 20 min. [^{14}C]PFA bound onto BBMV was determined by a standard rapid filtration technique [4].

Some of the tested compounds resulted in displacement of bound [^{14}C]PFA from BBMV; invariably, the displacement was more pronounced at a 15 mM concentration of the added compounds than at 2 mM (data not shown). From derivatives of PAA, only the α-Br-PAA showed a higher degree of [^{14}C]PFA displacement than PAA; hydrazido-PAA and hydroxylamido-PAA had a small but

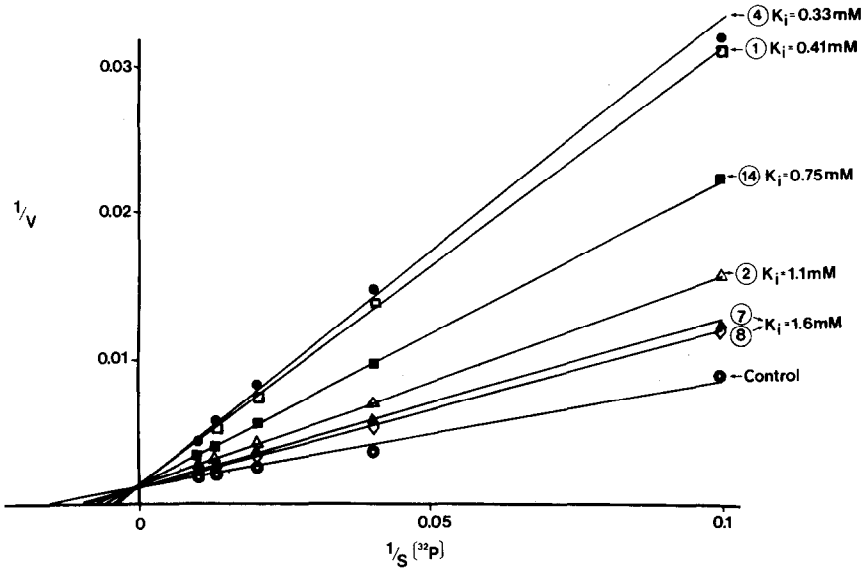

Fig. 2. Kinetics of inhibition of the initial Na^+-P_i gradient-dependent uptake of P_i by BBMV with phosphonate derivatives evaluated graphically by Lineweaver–Burk double-reciprocal plots. Each point is the mean of values from three experiments. All lines were fitted using the linear regression equation. The $^{32}P_i$ uptake was measured in the presence of $[Na_0^+ > Na_i^+]$ gradient in the initial (5 sec) uptake period and is expressed in nanomoles $^{32}P_i$ per milligram of protein per 5 sec. *Control*: The apparent K_m for P_i transport without the addition of any compounds was 71 μM and the V_{max} was 0.847 nmol/mg protein/5 sec. Uptake in the presence of added compounds is denoted by lines with test compounds identified by numbers (as in Fig. 1) and calculated inhibitory constant K_i. The $^{32}P_i$ uptake was measured in the presence of (1) α-Br-PAA (1 mM); (2) PAA (2 mM); (4) PFA (1 mM); (7) hydrazido-PAA (2 mM); (8) hydroxylamido-PAA (2 mM); and (14) β-Cl-ethyl-phosphonic acid (1 mM). Shown is a representative graph of three experiments. None of the tested compounds altered the V_{max}; more detailed data are presented in Table 2. Abscissa: Reciprocal values of $^{32}P_i$ (μM); ordinate: 1/velocity = $^{32}P_i$ transport rate (pmol $^{32}P_i$/mg protein BBMV/5 sec).

Table 2. Kinetic parameters of the effects of monophosphates and related compounds on Na^+-gradient-dependent P_i uptake by BBMV

Additions	Concn (mM)	V_{max} (nmol/5 sec/mg protein)	K_m (μM)	K_i (mM)
None (control)	0	0.945 ± 0.09*	79 ± 4	
Compound:				
1 PFA	1	0.844 ± 0.141	179 ± 4†	0.43 ± 0.01
2 PAA	2	0.894 ± 0.128	126 ± 5†	1.20 ± 0.06
3 Arsonoacetate (AsAA)	2	0.914 ± 0.065	73 ± 10	
4 α-Br-PAA	1	0.837 ± 0.059	199 ± 8†	0.37 ± 0.02‡
5 α-Ethyl-PAA	2	0.874 ± 0.036	83 ± 7	
7 Hydrazido-PAA	2	0.810 ± 0.075	102 ± 6†	1.56 ± 0.05‡
8 Hydroxylamido-PAA	2	0.841 ± 0.095	98 ± 6	1.57 ± 0.05‡
9 Aminomethylphosphonate	2	0.942 ± 0.055	72 ± 6	
10 Hydroxymethylphosphonate	2	0.898 ± 0.063	73 ± 7	
11 Monochloromethylphosphonate	2	0.924 ± 0.046	96 ± 8	1.58 ± 0.09‡
14 β-Cl-ethyl-phosphonate	2	0.935 ± 0.045	152 ± 8†	0.69 ± 0.03‡

The initial Na^+-gradient-dependent $^{32}P_i$ uptake was measured (Na_0^+ = 100 mM; Na_i^+ = 0) at 5-sec period, with final concentrations of $^{32}P_i$ of 10, 25, 50, 75, and 100 μM. Kinetic constants were determined graphically by linear regression from Lineweaver–Burk double-reciprocal plots (see Fig. 2).

* Each value denotes mean ± SE, N = 3 experiments.

† Significantly different ($P < 0.05$ or higher degree of significance, group t-test) from controls.

‡ Significantly different ($P < 0.05$ or higher degree of significance, group t-test) from K_i value from PAA.

detectable displacing effect. On the other hand, α-ethyl-PAA, α-PCl-phenyl-PAA and arsonoacetic acid had no discernible effect on [^{14}C]PFA binding (Table 3).

To examine the relationship of the ability to inhibit the Na^+-gradient uptake of $^{32}P_i$ by BBMV and to displace [^{14}C]PFA from binding on BBMV, we compared these parameters under conditions when the ratios of the added tested compound to the transported solute ($^{32}P_i$) and to the pre-bound [^{14}C]PFA ligand were both 1:20 (Fig. 3). Results of these experiments are summarized in Fig. 3, expressed as

Table 3. Displacement of [^{14}C]PFA binding from BBMV by PAA derivatives and related compounds

Additions	[^{14}C]PFA bound* (nmol/mg protein/20 min)	Δ%†
None (control)	2.81 ± 0.06	
Compound:		
1 Phosphonoformic acid (PFA)	1.55 ± 0.17‡	−45 ± 5§
2 Phosphonoacetic acid (PAA)	2.11 ± 0.08‡	−25 ± 3§
3 Arsonoacetic acid (AsAA)	2.80 ± 0.05	ND
4 α-Bromo-phosphonoacetic acid (α-Br-PAA)	1.97 ± 0.08‡	−30 ± 3§
5 α-Ethyl-PAA	2.80 ± 0.02	ND
6 α-*p*-Chloro-phenyl PAA	2.77 ± 0.03	ND
7 Hydrazido-PAA	2.22 ± 0.09‡	−21 ± 2§
8 Hydroxylamido-PAA	2.52 ± 0.09	−19 ± 2§
9 Aminoethylphosphonic acid	2.74 ± 0.07	ND
10 Hydroxymethylphosphonic acid	2.80 ± 0.02	ND
11 Monochloromethylphosphonic acid	2.33 ± 0.04‡	−17 ± 2§
12 Dichloromethylphosphonic acid	2.80 ± 0.06	ND
13 Trichloromethylphosphonic acid	2.84 ± 0.04	ND
14 β-Cl-Ethyl-phosphonic acid	1.90 ± 0.03‡	−32 ± 3§

BBMV were preincubated with 0.8 mM [^{14}C]PFA in a MTH medium containing 100 mM NaCl for 20 min at 20°. Thereafter, various test compounds dissolved in the same medium were added (in a final concentration of 15 mM), incubation was continued for another 20 min at 20°, and then the [^{14}C]PFA bound was determined (in nmol [^{14}C]PFA/mg protein).

* Each value denotes mean ± SE, N = 3 experiments.

† Percent difference from the control value (no additions), taken as 100%.

‡ Significantly different from control ($P < 0.01$ or higher degree of significance, group *t*-test).

§ Significantly different from controls ($P < 0.05$ or higher degree of significance, paired *t*-test).

a percent of the control value. The analysis indicates a very close correlation ($r = 0.925$, $N = 12$; $P < 0.001$, *t*-test) between the ability of test compounds to inhibit P_i uptake and to displace [^{14}C]PFA from binding on BBMV (Fig. 3).

DISCUSSION

Experimental examination of the effects of chemical derivatives of phosphonoacetic acid (PAA) and several structurally related relevant compounds provides several new insights concerning requirements of organic compounds containing a phosphonyl radical for binding on Na^+-P_i cotransporter in renal BBM and for its resulting effect, i.e. inhibition of Na^+-P_i cotransport [1, 7]. All derivatives tested in this study showed a strictly competitive inhibitory effect, suggesting that all these active compounds were bound on Na^+-Pi_i cotransporter in BBM, most likely through the phosphono-moiety in the molecule [1, 4, 7]. As suggested in our previous studies, this Na^+-P_i binding site is a locus on the Na^+-P_i cotransporter facing the luminal external face of BBM [4, 7]. The requirement for the size of the phosphonyl group can be quite strict. Namely, arsonoacetic acid even in the highest concentration tested (Tables 1 and 2) showed no inhibitory effect on Na^+-P_i cotransport or on [^{14}C]PFA binding (Table 3). Although arsenic acid is a rather weak competitive inhibitor of Na^+-P_i transport [15] and at a higher concentration also displaced [^{14}C]PFA binding from BBMV [4], apparently the arsono group linked to the carbon moiety, as in AsAA, has a lower or no capacity to interact with the Na^+-P_i binding site on BBM.

From a comparison of the actions of PAA and its several derivatives a few tentative conclusions can be deduced. Apparently the electronegativity of the carboxylic group in the PAA molecule is an important feature for binding and inhibitory action, since decreases of electronegativity by substitution with a hydroxylamino group or a hydrazido group markedly decreased effectivness of these derivatives (Tables 1–3). The fact that electronegativity *per se* rather than specifically the carboxylic group is required may be suggested by the finding that β-Cl-ethyl-phosphonic acid, which lacks a carboxylate group but in which electronegativity is conferred by the chloride atom, showed substantial inhibitory and binding capacity, much higher than PAA itself (Table 2, Fig. 2).

Perhaps the most interesting information comes from a comparison of the substitutions of PAA and the α-carbon. Replacement of one hydrogen by either an aliphatic ethyl group or an aromatic *p*-Cl-phenyl-group in PAA resulted in a total loss of inhibitory and binding activity, whereas the α-substitution with Br much increased both activities (Tables 2 and 3). From these comparisons, we can surmise that relatively bulky substituents on the α-carbon cause a steric hindrance for the access of phosphonyl radical to binding pocket on the Na^+-P_i cotransporter, and thus renders these compounds inactive. On the other hand, α-C substitution with a relatively small Br atom did not have a detrimental effect. On the contrary, the addition of Br enhanced the electronegativity of the modified PAA molecule and also increased affinity of this derivative for Na^+-P_i cotransporter (Tables 2 and 3). This notion is also in accord with our recent observation that a similar

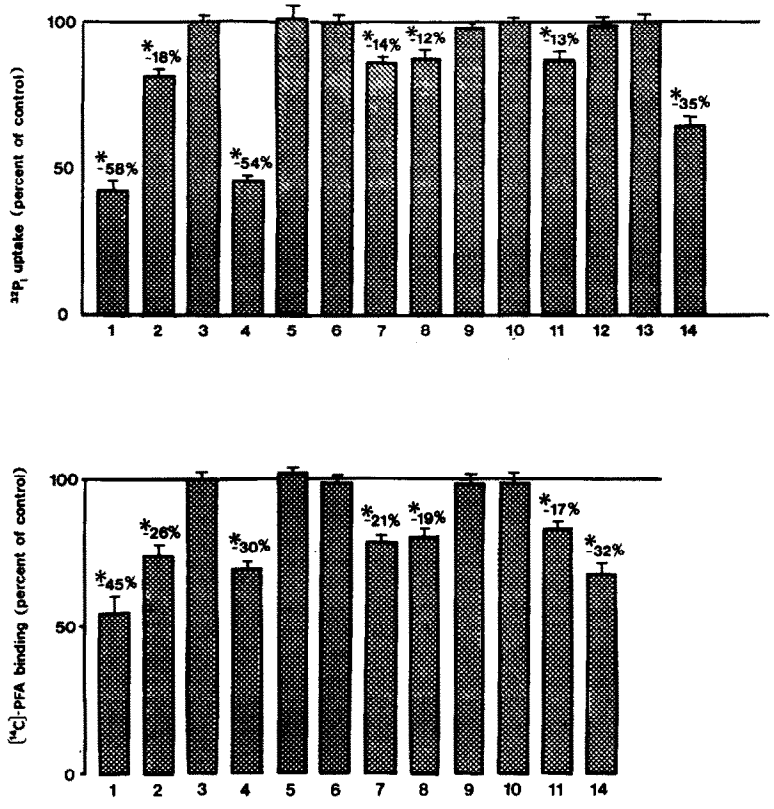

Fig. 3. Relationship between the inhibition of Na^+-gradient-dependent transport of P_i by BBM and displacement of [^{14}C]PFA bound on BBMV by phosphonate derivatives. *Upper panel:* Effect of monophosphonates and related compounds on Na^+-gradient-dependent uptake of $^{32}P_i$-phosphate. The uptake was measured at the initial 10-sec phase; the concentration of $^{32}P_i$ was 0.1 mM, and test compounds were added in final concentrations of 2 mM ($^{32}P_i$-to-inhibitor ratio was 1:20). The data are expressed as percent of the control uptake (0.742 ± 0.05 pmol $^{32}P_i$/mg protein/10 sec, N = 3) without addition of compounds, taken as 100%. Each bar represents the mean ± SE of three (N = 3) individual experiments. An asterisk (*) denotes significant inhibition ($P < 0.05$, or higher degree of significance, paired *t*-test). *Lower panel:* Displacement of [^{14}C]PFA bound on BBMV (in the presence of 100 mM NaCl) by monophosphonates and related compounds. The experimental design is described in detail in the legend to Table 3. The final concentration of [^{14}C]PFA was 0.8 mM and that of the added compounds was 15 mM (ligand-to-phosphonate ratio 1:19). The control value (without additions) was 2.81 ± 0.06 nmol [^{14}C]PFA/mg protein. The effect of the added test compounds is expressed as a percent difference from the control value taken as 100%. The bars denote mean ± SE of three (N = 3) experiments. An asterisk (*) denotes significant inhibition ($P < 0.05$ or higher degree of significance, paired *t*-test). The percent displacement by compared tested compounds (compounds 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 14) was closely correlated with percent inhibitory effect ($r = 0.925$, N = 12; $P < 0.001$, *t*-test).

PAA derivative, α-Cl-α-Br-PAA [14], is a more effective inhibitor than PAA, even more potent than α-Br-PAA. Its higher inhibitory potency is due, as in the case of α-Br-PAA (Fig. 2), to an increase in apparent affinity to the Na^+-P_i cotransporter [14].

Finally, comparison of β-Cl-ethyl-phosphonic acid with methylphosphonic acid derivatives should also be briefly considered. Aminomethylphosphonic and hydroxymethylphosphonic acid showed no inhibitory effect (Tables 1 and 2) or binding capacity (Table 3), whereas monochloromethylphosphonic acid showed both some Na^+-P_i transport inhibitory activity and also small but significant displacement of [^{14}C]PFA binding (Tables 1 and 3). However, a comparison with the effects of β-Cl-ethyl-phosphonic acid indicates that the latter has a much higher affinity towards Na^+-P_i cotransporter, i.e. more than two times lower K_i (Table 2). This suggests that the greater distance of Cl from the phosphonyl moiety, perhaps for steric reasons, increases the affinity of the phosphonyl group or its accessibility to the Na^+-P_i binding site.

Finally, the finding of a close and direct quantitative correlation between the [^{14}C]PFA binding displacement capacity of phosphonate derivatives and their ability to competitively inhibit Na^+-P_i cotransport (Fig. 3) lends further strong argument for the hypothesis [1, 4] that monophosphonates, namely phosphonocarboxylates, inhibit Na^+-P_i cotransport by competing, in the presence of Na^+, with P_i for P_i-binding site at the luminal side of the Na^+-P_i cotransporter [1, 4, 7].

In conclusion, our present observations suggest several features and principles indicating how the phosphonate inhibitors of Na^+-P_i cotransporter derived from PFA and PAA should be perfected in

designing new potent competitive inhibitors of this Na^+-P_i cotransport system in both renal and intestinal BBM, and which will have preserved specificity and considerably higher affinity than PFA and PAA. Our recent finding on the effect of α-Br,α-Cl-PAA indeed suggests that these guidelines can be applied successfully [14]. The development of specific, potent and nontoxic inhibitors of Na^+-P_i cotransport is a highly desirable goal for both theoretical and applied studies of this cotransport system [1, 4, 7].

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