Phenylphosphonate: A $^{31}$P-NMR Indicator of Extracellular pH and Volume in the Isolated Perfused Rabbit Bladder

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$^{31}$P-NMR has been used extensively to estimate intracellular pH. It also can be used to measure extracellular pH and volume when an NMR-detectable extracellular phosphorous probe is used. Phosphonic acids have been suggested as useful $^{31}$P-NMR extracellular markers. The present study was designed to assess the utility of phenylphosphonic acid (PPA) as a $^{31}$P-NMR extracellular marker in perfused smooth muscle. Rabbit bladder strips were exposed to PPA concentrations of 1–20 mM. Tension development in response to maximal carbachol challenges (10 $\mu$M) was independent of PPA concentration. Addition of PPA (6 mM) to the perfusate supplying the isolated resting rabbit bladder had no effect on $^{31}$P-NMR-detectable phosphatic compounds. PPA's resonance frequency was distinctly downfield from endogenous phosphates and demonstrated a pH-dependent chemical shift of ± 1.12 ppm/pH unit over the range of 6.4 to 7.6 with a pK' of 7.09 at 23°C. The time courses for washing PPA in and out of the resting bladder were best described by monotonic exponential growth ($r = 0.972; n = 3$) and decay ($r = 0.972; n = 3$) equations, respectively. Rate and time constants for PPA wash-in (0.039 ± 0.004 min$^{-1}$ and 25.7 ± 2.3 minutes) and washout (0.038 ± 0.000 min$^{-1}$ and 26.3 ± 0.0 minutes) were not significantly different. Using steady state PPA and ATP peak intensities and concentrations, an extracellular-to-intracellular ratio was calculated to be 0.31 ± 0.03 (n = 3). These data indicate that PPA remains distributed exclusively in the extracellular spaces. Further, they affirm that PPA is noncytotoxic and pH-sensitive over a physiological pH range under normal flow conditions. Using PPA, therefore, is a reliable way to measure both extracellular pH and volume while concomitantly monitoring the energy state of the cell. These measurements will assist in understanding the dynamic interdependence of extracellular and intracellular metabolic events. (Circulation Research 1987;60:472-477)
Materials and Methods

Bladder Dissection

Male New Zealand white rabbits (2–3 kg) were anesthetized with ketamine hydrochloride (55 mg/kg i.m.) and subsequent infusions of sodium pentobarbital (15 mg/kg i.v.). Cautery was used to open the lower abdominal wall, and then the small intestine was retracted to expose the bladder and its vasculature. Cavai and aortic branches supplying caudal structures other than the bladder were ligated and cut. The aorta was cannulated (PE-100 tubing), and the inferior vena cava was cut to allow free drainage of the perfusate. The empty bladder was removed from the animal with its vasculature intact to the level of the aorta. Measurements were made with the tissue at ambient temperature (23–24°C).

Perfusion Techniques

The perfusate contained NaCl 116 mM, NaHCO$_3$ 24 mM, KCl 4.6 mM, KH$_2$PO$_4$ 1.2 mM, CaCl$_2$ 2.5 mM, MgSO$_4$ 1.2 mM, glucose 7 mM, sodium pyruvate 0.15 mM, 10 µg/ml gentamicin sulfate, 35 mg/ml bovine serum albumin, and 8–10% (vol/vol) of washed rejuvenated dog or human erythrocytes.  Also, it contained 6 mM PPA (Aldrich Chemical Co., Milwaukee, Wis.) titrated to pH 7.2 during wash-in periods of 21.  It was typically 30–35 Hz. The coil was then tuned to the proton line width before line broadening was applied. The NMR probe holding the perfused bladder was inserted in the superconducting magnet (field 9.4 Tesla) of a Bruker (Billerica, Mass.) AM 400 wide-bore (9 mm) spectrometer interfaced with an Aspect 3000 computer. Either a standard 20-mm multinuclear broadband probe or a 10-mm $^{31}$P-dedicated probe accommodating a 4-turn Helmholtz configuration coil was used. The Helmholtz coil was constructed from 10-gauge Formvar-coated copper wire. Using the proton signal from the bladder, room temperature shim coils were adjusted to improve magnetic field homogeneity. The proton line width before line broadening was typically 30–35 Hz. The coil was then tuned to the $^{31}$P resonance frequency of 161.9 MHz, and spectra were collected at 1-second intervals using the Fourier transform mode. Between 480 and 720 free induction decays (FIDs; i.e., 8–12-minute accumulations) were averaged in each experiment, depending on the probehead and the size of the muscle. Pulse width, determined by optimizing the phosphocreatine (PCr) signal-to-noise ratio, was 8–8.3 microseconds or 10–27 microseconds (using a 1-second delay) for the 10- and 20-mm probes, respectively. Spectral width was 9,000 Hz using quadrature detection and 2K data points/scan.

NMR spectra were analyzed using the Bruker DISB851 programs for determining peak positions and integrals. Prior to Fourier transformation, the summed FIDs were multiplied by a line-broadening constant corresponding to the inherent PCr signal line width (typically 20–25 Hz). The transformed spectra were then plotted and signal frequencies recorded as parts per million (ppm) chemical shift (δ) relative to the PCr peak position (assigned −2.52 ppm). Peak integrals were determined after spline baseline-correction of the spectra. Longitudinal relaxation rates (T1) for phosphorylated compounds in the bladder were determined using progressive saturation. The T1 for PPA in samples of perfusate was determined using the inversion recovery method.

Intracellular pH data were determined from the chemical shift of Pi (δ-Pi) relative to PCr. The equation:

$$\text{pH}_{\text{ic}} = 6.77 + \log \left[ \frac{(0.89 - \delta-\text{Pi})}{(\delta-\text{Pi} - 3.19)} \right]$$

(1)

described the pH titration curve for Pi derived from spectra of samples of mock intracellular fluid. 19 Estimations of intracellular free magnesium (Mg$^{2+}$) levels were based on the β-ATP peak position relative to PCr (δ-ATP) and the spectroscopically-derived Mg$^{2+}$ titration curves for ATP defined by

$$\text{pMg} = 4.108 + \log \left[ \frac{(-18.38 - \delta-\text{ATP})}{(\delta-\text{ATP} + 21.54)} \right]$$

(2)

for pH 7 and

$$\text{pMg} = 4.036 + \log \left[ \frac{(-18.38 - \delta-\text{ATP})}{(\delta-\text{ATP} + 21.71)} \right]$$

(3)

for pH 6.6. 22

A pH titration curve was constructed for PPA to ensure its pH-sensitive range. PPA (6 mM) and PCr (3 mM) were added to perfusate samples. Samples were titrated with aliquots of 1N NaOH buffer to final pH values between 5.6 and 8.4. Spectroscopic analyses of PPA peak positions relative to PCr were conducted at room temperature, and the data were fit to:

$$\text{pH}_{\text{ic}} = \text{pK}' + \log \left[ \frac{\delta-\text{PPA}}{(A - \delta-\text{PPA})/\text{pK}'} \right]$$

(4)

where δ-PPA is the measured PPA peak position and A and B are the PPA chemical shift end points measured at low and high pH, respectively.

Bladder Strip Preparations

The bladder was removed from the rabbit (anesthetized with ketamine hydrochloride, 55 mg/ml i.m., and sodium pentobarbital, 15 mg/kg i.p.) and immersed in cold Ringer’s solution. Longitudinal muscle strips were dissected from the outer surface. One end of each strip was attached to a Kulite strain gauge and...
Figure 1. Effect of phenylphosphonate (PPA) on $^{31}$P-NMR-detectable intracellular phosphagens. A relaxed rabbit bladder was perfused at 0.42 ml/min before (Panel A) and after (Panel B) PPA (6 mM) was added to the perfusate. Panel C: Difference spectrum for before and after PPA. The numbered peaks are identified in Table 1. NS = number of scans; RD = time between each scan; and LB = line broadening constant used for exponential multiplication.

Statistical Analyses

Data are presented as means and standard errors of the mean (SEM). A paired t test was used to evaluate differences between PPA wash-in and washout data. Dunnett's procedure was used to judge significance of observed differences between treatment and control tensions from bladder strip experiments. A 0.05 level of significance was chosen to test the null hypothesis.

Results

The spectrum of resting rabbit bladder perfused at 0.42 ml/min shown in Figure 1A has 7 naturally occurring resonance bands representing chemically different phosphate groups. These compounds have been identified previously by high performance liquid chromatography (HPLC) and enzymatic analyses of bladder extracts. The primary signal (i.e., the compound present in the highest concentration) and chemical shift for each peak are listed in Table 1. Chemical shifts were determined relative to the conventional standard of 85% inorganic orthophosphate with endogenous PCr serving as the internal standard ($8$-PCr = $-2.52$ ppm). In addition to the primary signal, several of the peaks reflect signal contributions from compounds present in relatively low concentrations. For example, peaks 1 and 3 include phosphocholine and phospho-glycerol-ethanolamine, respectively; peaks 5 and 6 contain signals from the phosphoryl resonances of ADP, and peak 6 has a prominent upfield shoulder due to NAD and UDP-glucose; and peak 7 reflects signal contributions from UTP and GTP, accounting for 22% of the total peak area. Intracellular pH and free Mg$^{2+}$

Table 1. $^{31}$P-NMR Chemical Shifts of Resonances in Resting Perfused Rabbit Bladder

<table>
<thead>
<tr>
<th>Peak</th>
<th>Phosphatic compound</th>
<th>Chemical shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phosphoethanolamine (PE)</td>
<td>3.81 ± 0.09</td>
</tr>
<tr>
<td>2</td>
<td>Inorganic phosphate (Pi)</td>
<td>2.51 ± 0.07</td>
</tr>
<tr>
<td>3</td>
<td>Glycerophosphorylcholine (GPC)</td>
<td>0.60 ± 0.02</td>
</tr>
<tr>
<td>4</td>
<td>Phosphocreatine (PCr)</td>
<td>-2.52 (ref.)</td>
</tr>
<tr>
<td>5</td>
<td>γ-ATP</td>
<td>-5.01 ± 0.06</td>
</tr>
<tr>
<td>6</td>
<td>α-ATP</td>
<td>-10.12 ± 0.06</td>
</tr>
<tr>
<td>7</td>
<td>β-ATP</td>
<td>-18.86 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SEM ($n = 7$) for chemical shifts (8 ppm) of cytosolic phosphorous-containing compounds. Means represent the average of 1,800 1-second scans. Measurements were made while resting bladders were perfused at 0.42 ml/min.
content of the perfused resting bladder, defined by the chemical shifts of peaks 2 and 7, respectively, were 7.15 ± 0.06 and 0.45 ± 0.02 mM (n = 7).

The spectrum shown in Figure 1B was collected 1 hour after PPA (6 mM) was added to the perfusate. These data were collected from the same bladder as those shown in Figure 1A, using the same perfusion rate of 0.42 ml/min. PPA resonated conspicuously downfield (δ-PPA = 12.77 ± 0.02 ppm; n = 3) from endogenous phosphatic compounds and had no effect on intracellular pH or free Mg²⁺ levels (p > 0.05). Further, the difference spectrum shown in Figure 1C confirms that PPA did not alter levels of intracellular phosphagens or their chemical environment.

Results from bladder strip experiments (Figure 2) provided additional evidence that PPA does not alter the energy state of smooth muscle. Maximum tension generated in response to carbachol (10 μM) was 1.12 ± 0.17 x 10⁻⁵ N/m² under control conditions (viz, [PPA] = 0). Tensions generated when [PPA] > 0 mM were not significantly different from control (p > 0.05).

Data defining pH characteristics of PPA are shown in Figure 3. A pH titration curve for PPA was constructed over the pH range 5.6–8.4 using perfusate samples with PPA (6 mM). The position of PPA’s resonance band was dependent on pH over the range 6.4–7.6 and showed a pH-dependent chemical shift of ± 1.12 ppm per pH unit. PPA’s pk’ was 7.09, and its titration end points for the fully complexed (protonated) and dissociated species were 13.67 and 11.98 ppm, respectively. The equation

\[
\text{pH}_{\text{PCr}} = 7.09 + \log[(13.67 - \delta-\text{PPA})/(\delta-\text{PPA} - 11.98)]
\]

incorporates these data, defines the titration curve shown in Figure 3 between pH 5.6 and 8.4, and permits the determination of pH based on the observed δ-PPA.

Figure 4 shows a typical series of spectra depicting the time course for washing PPA through the bladder. PPA (6 mM) was added to and subsequently removed from the perfusate while perfusion flow was maintained at 0.42 ml/min. During these procedures, peak intensities for endogenous phosphate compounds, pH IC (7.09 ± 0.01), and free Mg²⁺ IC (0.54 ± 0.02) remained stable (p > 0.05; n = 3). When PPA was included in the perfusate, a PPA signal (SPPA) appeared within 20 minutes (PPA wash-in), then the signal increased to become stable within 40 minutes (steady state). After a 2–4 hour steady state, a PPA-free perfusate was used. SPPA disappeared from the spectrum within 40 minutes (PPA washout). The time course for the growth of SPPA during wash-in was best defined (r = 0.972; n = 3) by the equation

\[
S_{\text{PPA}} = (3.8 \pm 1.5)(1 - e^{0.039 \pm 0.004t})
\]

which describes a monoexponential growth curve. Similarly, the time course for the disappearance of SPPA during washout was best described (r = 0.972; n = 3) by the equation

\[
S_{\text{PPA}} = (25.5 \pm 2.7)e^{0.038 \pm 0.004t}
\]

which describes a monoexponential decay curve. The mean wash-in rate and time constants (0.039 ± 0.004 min⁻¹ and 25.7 ± 2.3 minutes, respectively) were not different from those for washout (0.038 min⁻¹ and 26.3 minutes, respectively). The pH IC determined from PPA during steady state in these experiments was 7.16 ± 0.02 (n = 3).

Since PPA and ATP are distributed entirely within the extracellular (EC) and intracellular (IC) spaces,
respectively, the relative size of the EC space can be determined using steady state peak intensities of PPA and β-ATP. Signal intensities (S) of NMR-detectable compounds are a product of concentration (C) and their volumes of distribution (V) within the magnetic field. Therefore, the equation

\[ \frac{S_{\text{PPA}}}{S_{\text{ATP}}} = \frac{(C_{\text{PPA}} \times V_{\text{EC}})/(C_{\text{ATP}} \times V_{\text{IC}})} \] (8)

after rearrangement

\[ \frac{V_{\text{EC}}}{V_{\text{IC}}} = \frac{(S_{\text{PPA}} \times C_{\text{ATP}})/(S_{\text{ATP}} \times C_{\text{PPA}})} \] (9)

calculates an index of EC volume, \( V_{\text{EC}}/V_{\text{IC}} \). The \( S_{\text{PPA}} \) and \( S_{\text{ATP}} \) are determined from T1-corrected steady state peak integrals; the T1s for PPA and ATP were 4.6 ± 0.1 (n = 5) and 1.1 ± 0.2 (n = 2), respectively. \( C_{\text{PPA}} \) is known, and \( C_{\text{ATP}} \) has been determined previously for isolated perfused bladders (\( C_{\text{ATP}} = 1.24 \) mM). Mean steady state \( V_{\text{EC}}/V_{\text{IC}} \) for resting rabbit bladder perfused at 0.42 ml/min was 0.31 ± 0.03 (n = 3).

**Discussion**

This report promotes the concept that PPA is a reliable nontoxic extracellular marker. Data in Figures 1 and 2 affirm that PPA is nontoxic to cells since both the tissue's energy state as well as its contractility remains stable during prolonged exposure to PPA. Data in Figure 4 indicate features of PPA which suggest that it is extracellularly distributed and maintained. The time courses for washing PPA in and out of the bladder were not only accurately characterized by monotonous exponential equations (6 and 7) but also were defined by equivalent time constants (25.7 ± 2.3 minutes for wash-in and 26.3 ± 0.0 minutes for wash-out; n = 3). These data imply that PPA was added to and removed from a single extracellular compartment as a nonbinding, nonmetabolizable molecule. Meeting such criteria is a primary requirement for any material to be used as an extracellular indicator.

PPA has an additional characteristic that makes it particularly suitable for \(^{31}\)P-NMR extracellular pH and volume measurements. Data in Figures 1B and 4 demonstrate that PPA establishes a unique spectral peak with a resonance frequency distinct from all endogenous phosphorous-containing compounds. The downfield position of the PPA signal relative to other cellular phosphorous signals is especially important since PPA's peak position is pH sensitive (Figure 3). Changes in the pH environment of PPA over a range compatible with cell viability do not result in peak overlap. This makes extracellular pH determinations using PPA unambiguous.

PPA's relatively isolated peak position also makes its integral determinations straightforward; minimal difficulty is encountered in delimiting the PPA peak for integration since it is not encroached on by neighboring signals. Similarly, the β-ATP signal is located slightly upfield from other endogenous peaks making its integral relatively easy to define. Consequently, extracellular-to-intracellular volume calculations (equations 8 and 9), which are based on PPA and β-ATP peak integrals and their concentrations, can be made with confidence.

Data reported here demonstrate that PPA is pH sensitive and remains distributed exclusively in the extra-
cellular spaces of perfused smooth muscle. They also clearly demonstrate that PPA is innocuous to smooth muscle metabolism and energetics. Consequently, using PPA provides a means of concomitantly measuring extracellular and intracellular pH and volume ratios as well as other dynamic cellular processes that may directly affect them. Additionally, changes in fluid distribution and/or ion gradients across cell membranes can be followed over time as oxygenation, osmotic shifts, perfusion flow rates, and other factors operate to change either cell membrane permeability on cellular metabolism. Such information can greatly assist in understanding the dynamic interdependence of extracellular and intracellular metabolic events.

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