31P NMR Spectroscopy, Chemical Analysis, and Free Mg2+ of Rabbit Bladder and Uterine Smooth Muscle*

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31P NMR spectra of isolated rabbit bladder and uterus were obtained under steady-state arterial perfusion in vitro at rest and while stimulated. The spectra contained seven major peaks: phosphoethanolamine, sn-glycer(3)phosphocholine, inorganic phosphate (P), phosphocreatine, and the γ, α, and β peaks of ATP. Chemical analyses, high-pressure liquid chromatography, and NMR spectroscopy of aqueous extracts of bladders identified a number of other components that also made contributions to, but were not resolved in, the spectra of the intact tissues: UTP, GTP, UDP-Glc, NAD+, phosphocholine, and sn-glycer(3)phosphoethanolamine. Intracellular pH of unstimulated bladders and uteri, measured from the chemical shift of the P, peak, was 7.10 ± 0.09 S.D. and 7.01 ± 0.12 S.D., respectively. The chemical shift of the β-ATP peak in the smooth muscles was significantly upfield (−0.3 ppm) compared to the chemical shift observed in striated muscles (cat biceps and rat myocardium). An ADP peak was identified in stimulated and ischemic bladders. The chemical shifts of the nucleotides observed in perfused bladders were calibrated as a function of free Mg2+ concentration in solutions containing phosphocreatine, P, ADP, and ATP at an ionic strength of 180 mM. We derived the following estimates for the intracellular free Mg2+ concentration: uterus, 0.40 mM; unstimulated bladder, 0.46 mM; stimulated and ischemic bladder, 0.50 mM (from the ATP chemical shift) and 0.45 (from the ADP chemical shift); cat biceps, 1.5 mM; and rat myocardium, 1.4 mM.

NMR spectroscopy has been used extensively to probe the intracellular milieu of a variety of cells and isolated organs, particularly of skeletal and cardiac muscle (1-6). 31P spectroscopic studies have provided detailed and accurate information on the metabolic and energetic states and on the intracellular pH of these striated muscles at rest, during and following various degrees of activation, and under pathological conditions.

We have developed two arterially perfused preparations of smooth muscle as in vitro experimental models: the rabbit uterus and urinary bladder. We report here the results of our studies on these smooth muscles, at rest and during metabolic stress caused by maximal pharmacological and ischemic stimulation. We focus on the comparison between 31P NMR spectral and conventional chemical analyses, and on the interpretation of the chemical shifts of the nucleotides as a probe of the intracellular Mg2+ concentration. Our results also extend the results of previous studies of smooth muscle preparations (7-13) in several ways. First, in all of the previous reports, a large peak was noted downfield of P in the monophosphate ester region of the spectrum, sometimes described as a sugar phosphate. Yet in no reported chemical analysis of smooth muscle metabolite content has any significant amount of hexose or other sugar phosphate been found (14, 15). We have identified this spectral peak by a number of independent means as phosphoethanolamine (PEtn).

Second, because both the presence and absence of a significant peak intensity (7-13) have been reported, and because P may serve as an important metabolic regulator, we made a quantitative analysis of the metabolites present in the rabbit bladder, both by 31P NMR methods and by chemical and enzymatic analyses of tissue extracts. With the exception of the work of Hellstrand and Vogel (7), no direct comparison of results by NMR and chemical methods has been carried out.

Finally, the Ca2+ sensitivity of the smooth muscle actomyosin ATPase (22) and of smooth muscle mechanical properties (23-25) are a function of the Mg2+ concentration. In addition, the Mg2+ content of smooth muscle has been shown to be dependent on estrogen treatment in the rat uterus (12). We used the chemical shift of the β peak of ATP to determine its degree of complex formation with Mg2+ ion (16-21), thus probing the intracellular free Mg2+ concentration. We estimate the free Mg2+ concentrations to be significantly lower in smooth than in striated muscle. A preliminary report of this work has been given (26).

EXPERIMENTAL PROCEDURES

Animals—Experiments used virgin female New Zealand White rabbits weighing between 2 and 4 kg; one male was also used. Those rabbits from which the uteri were obtained were injected subcutaneously for 3 weeks prior to the experiment with 200 µg/day estradiol 3-benzoate (Sigma) dissolved in corn oil (Mazola) to produce uterine hypertrophy (27).

Surgical Procedures—The rabbits were anesthetized by intraperi...

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1 The abbreviations used are: PEtn, phosphoethanolamine; GroPCo, sn-glycer(3)phosphocholine; PCr, phosphocreatine; FID, free induction decay; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; NTP, nucleotide triphosphate; HPLC, high-pressure liquid chromatography.
tonal injection with 60 mg/kg pentobarbital. When deep surgical anesthesia was achieved, the ventilation of the animals was controlled by a respirator. A midline incision in the abdominal wall was made, and the appropriate organ was dissected from the aorta with its arterial structures intact, as follows. The left horn of the uterus was intact, as follows. The left horn of the uterus was the left femoral artery, the left vaginal artery, the left hemorrhoidal column (Waters Associates). PEtn was separated from ethanolamine, the column elution buffer. HPLC used a sulfonic acid cation exchange by a respirator. A midline incision in the abdominal wall was made, anesthesia was achieved, the ventilation of the animals was controlled and the appropriate organ was dissected from the aorta with its branches were tied and did not leak. The perfused hemi-uterus was then removed from the animal and mounted on the NMR probe. The surgical procedure was similar for isolating the circulation to the bladder, except that its right and left arterial supplies were preserved by separately tying branches leading to the large intestine, to the left and right horns of the uterus, and to the vagina. For both the perfused bladder and hemi-uterus it was necessary to maintain the normal anatomical topology and to avoid tension and twisting of the arteries and veins of the preparation on the probe, in order to allow good perfuse flow. The urine in the bladder was removed and the bladder was rinsed with isotonic sodium chloride. The animal was subsequently killed by a large dose of pentobarbital.

Perfusion—An erythrocyte-containing perfusate was employed, prepared and used in the same way as with our standard perfusate, except that it contained no papaverine. The perfusate was equilibrated with 95% O₂, 5% CO₂, and pumped at a constant flow of 0.4–0.6 ml/min to a 2–3-g muscle preparation (28). The perfusate had an average hematocrit of 13%, and with gas equilibration with 95% O₂, 5% CO₂ at 55 °C, an oxygen content of 4 mm, and a pH of 7.25. The flow was sufficient to maintain a perfusion pressure in the range of 25–40 torr, and to deliver oxygen to a typical 2-g preparation at a rate of 1 µmol of oxygen/min. This rate of oxygen delivery is 10-fold in excess of the maximum oxygen consumption reported for vascular smooth muscle (14). The temperature of the perfused preparation on the NMR probe, which was magnetically shielded in a Magnebath (Sigma) was freshly prepared in Ringer’s solution and added to the perfusate when stimulation of the preparations was required.

Chemical Analysis—When the desired NMR spectra were obtained, the bladders and uteri were frozen between the plates of a brass clamp precooled in liquid nitrogen, without interrupting the perfusion. The frozen tissues were stored at −80 °C until extracted for analysis. The frozen tissues were weighed without thawing, pulverized in liquid nitrogen, and extracted for subsequent analysis in a solution of 3.5% (w/v) perchloric acid and 20% (v/v) ethanol, with a volume ratio of tissue to extraction solution of 1:8 (29). The extracts were neutralized with 3 M KOH, 0.5 M triethanolamine solution until the pH was just greater than 7. The precipitated KClO₃ was removed and the extract supernatant was stored at −80 °C in several aliquots for use in chemical and HPLC analyses. For NMR spectral analyses of extracts, similar supernatants were obtained from unperturbed bladders rapidly frozen after dissection and the diluent 20-fold in water and treated batchwise three times with Chelex 100 (Bio-Rad) to remove paramagnetic and other cations. The treated extract was lyophilized and reconstituted in a small volume of water containing 20% (v/v) D₂O.

High-pressure liquid chromatography (HPLC) was used for nucleotide analyses by a modification of the method of Swain et al. (30) using a strong anion exchange column (Partisil column, Altex, Berkeley, CA). The eluent was pumped at 40 ml/h to form a nonlinear gradient between 4 mM NH₄H₂PO₄, pH 2.8, in 10% (v/v) acetonitrile and 750 mM NH₄H₂PO₄, pH 3.8, in 10% (v/v) acetonitrile. Peaks were quantified by optical absorption at 254 nm in comparison with known standards. Chemical identification of the eluted peaks was proven by optical spectral analysis.

For analysis of phosphoethanolamine and related amino compounds, tissue samples were homogenized at 80–90 °C in 0.1% trifluoroacetic acid solution. The urine in the bladder was also re-extracted at 80 °C in 0.1% trifluoroacetic acid, 20% methanol. After centrifugation the supernatants were treated with Sep-Pak C₈ cartridges (Waters Associates), lyophilized, and dissolved in the column elution buffer. HPLC was a sulfonic acid cation exchange column (Waters Associates), but it was adapted from ethylene diamine, sn-glycerol(3)phosphoethanolamine, CDP-ethanolamine, and sn-glycerol(3)phosphoserine using an isocratic elution buffer containing 67 mM citric acid, pH 2.5, at room temperature and a flow rate of 20 ml/h. The primary amines in the column eluate were detected by optical absorption at 330 nm after post-column derivatization with o-phthalaldehyde reagent (Pickering Laboratories, Mountain View, CA).

With the exception of inorganic phosphate, the other metabolites were assayed using the fluorometric methods of Lowry and Paozscheau (32). Creatine was assayed enzymatically by the production of uric acid (absorption maximum at 293 nm) in a cuvette containing 50 mM triethanolamine, pH 7.6, 3.75 mM EDTA, 0.56 mM inosine, 0.03 unit/ml xanthine oxidase, 0.16 unit/ml purine nucleotide phosphorylase, and 10–100 µM/µl extract.

The tissue magnesium content was measured in the perchloric acid extracts (diluted 1:50) by atomic absorption analysis (Perkin-Elmer model 290) at 2025 nm.

The data are given as the mean value ± 1 S.D. unless stated otherwise.

Nuclear Magnetic Resonance Spectroscopy—Spectra were obtained in a Bruker HX-270 spectrometer, operating in the Fourier transform mode at a frequency of 109.3 MHz for 31P. A Bruker 10 mm probe was used to obtain spectra of solutions. For the uterus and bladder preparations, a probe was designed to hold the preparation while being continuously perfused in the middle of a single-turn Helmholtz coil as described (28). The preparations, which had widths of about 7–10 mm, were wrapped in Parafilm to maintain radiofrequency isolation of the moist surface of the preparation from the coil. This wrap also prevented tissue drying and directed the perfusate from the preparation to the base of the probe, from which it was continuously pumped. The probe was re-tuned to 109.3 MHz with each perfused preparation mounted in place. This assembly was shimmed on the 'H signal of the preparation; typically, proton line-widths were 40–50 Hz (≈0.4 ppm). The 90° pulse time was typically 25 µs. The free induction decays (FIDs) were filtered before Fourier transformation at the inherent line-width of the 31P PCr signal, typically 25 Hz. T1 times, determined both by inversion recovery and by progressive saturation, gave indistinguishable values and are averaged in Table IV. The addition of D₂O to the extracts and a deuterium lock was used to obtain high-resolution spectra. Because sn-glycerol(3)phosphocholine (Gro/Cho) was identified (see Results) in both the uteri and bladders, and because its chemical shift is insensitive to pH, it provided a convenient internal standard, with a chemical shift of 0.49 ppm with respect to 85% phosphoric acid at 0 ppm.

The protocol for obtaining spectra from the perfused preparations was (a) to measure the 90° pulse; (b) to take a fully relaxed control preparation with the 90° pulse time at 15–20°; (c) when required, to record spectra of stimulated or ischemic bladders at 3- or 4-s intervals using a 70° pulse, or at 1-s intervals using a 60° pulse; and (d) to repeat fully relaxed control spectra throughout the course of the experiment.

In order to achieve the largest rate of energy utilization during stimulation, the bladders were stretched (in the direction of the axis from the urethral outlet to the apex) in the NMR probe to a length which gave the maximal isometric force when stimulated by addition of carbachol to the perfusate. This length was 2.4 times the unrestrained length of the perfused bladder. The dose-response curve of maintained steady-state isometric force versus carbachol concentration at that length showed that a maximal contractile response was obtained at a concentration of 3 × 10⁻⁴ M (data not shown). Carbachol was used instead of acetylcholine because its stimulatory effect persisted in ischemia, whereas acetylcholine was rapidly hydrolyzed.

Calibration of the Chemical Shifts of P, and of ATP—The chemical shift of P, as a function of pH was measured in situ in a solution designed to mimic the intracellular ionic environment of smooth muscle. 5 mM MgATP, 5 mM PCr, 5 mM EGTA, 5 mM potassium phosphate, 100 mM KCl at pH 7.4 (the approximate conditions in the probe). A deuterium lock was used to obtain a calculated ionic strength of 190 mM. The predominant anion was propionate. The components of this solution were mixed according to the results of a multiple equilibrium calculation, using the association constants given in Table I for solutions at 25 °C. This method computes an apparent association constant (Kapp) as a function of the sum of all of the products and reactants (31-33), where [Me] is the concentration of H⁺ or Mg²⁺, [ligand] is the summed concentration of all of the chemical forms of the conjugate ligand (phosphate, ATP, ADP, PCT, EGTA, chloride, and imidazole), and [Me ligand] is the summed concentration of all of the relevant
published association constants are usually concentration constants, where $pX$ is the negative log of the concentration (of $H^+$ or $Me^+$), and solution-mixing procedure was used to construct curves of the desired pH; adding those amounts yielded solutions with a measured $pK'$, which is the negative logarithm of the apparent association constant, not activity constants, and because pH is a measure of activity. This concentration at pH 7.0 and the shift of Pi as a function of pH were fitted to the following equation:

$$pX = pK' + \log \frac{d_a - d_{obs}}{d_{obs} - d_b}$$

All chemical shifts were referenced to that of PCr present in the solutions at -2.52 ppm with respect to phosphoric acid at 0 ppm. All data are at 25 °C. Titration is between species given in column A and column B for the reaction $A \leftrightarrow B + H$ (or $Me^+$). Correct charge balance has been ignored for the components in Columns A and B.

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>$d_a$</th>
<th>$pK'$</th>
<th>$d_b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H_2PO_4$</td>
<td>$HPO_4^-$</td>
<td>0.88</td>
<td>6.77</td>
<td>3.19</td>
</tr>
<tr>
<td>$H_2ATP$</td>
<td>$ATP$ + $Me^+$</td>
<td>-18.38</td>
<td>4.11</td>
<td>-21.54</td>
</tr>
<tr>
<td>$H_2ATP$</td>
<td>$ATP$ + $Me^+$</td>
<td>-18.38</td>
<td>4.04</td>
<td>-21.71</td>
</tr>
<tr>
<td>$H_2ATP$</td>
<td>$ATP$ + $Me^+$</td>
<td>-18.38</td>
<td>4.04</td>
<td>-21.71</td>
</tr>
<tr>
<td>$H_2ATP$</td>
<td>$ATP$ + $Me^+$</td>
<td>-18.38</td>
<td>4.04</td>
<td>-21.71</td>
</tr>
</tbody>
</table>

The equation used to fit the data was:

$$pX = pK' + \log \frac{d_a - d_{obs}}{d_{obs} - d_b}$$

The spectral positions of the major peaks in the bladder and single uterine horns. Normally the spectra did not detectably change during the course of perfusion, typically lasting 10 h. The few preparations in which the series of control spectra did not show such stability (declining peak intensities and increasing peak widths) were not used further; those muscles were edematous and often contained focal hemorrhages. The predominant components of the seven labeled peaks are: peak 1, PEtn; peak 2, GroPCho; peak 3, GroPCho; peak 4, PCr; peaks 5, 6, and 7, respectively, the $\gamma$, $\alpha$, and $\beta$ peaks of ATP. The details for the chemical identification of the components of the peaks are described in the next section.

The spectral positions of the major peaks in the bladder spectra are representative of those obtained from rabbit bladders and single uterine horns. The NMR spectra of rabbit urinary bladder (bottom). The abscissa scale is in parts/million relative to 85% phosphoric acid, with the secondary standard GroPCho (peak 3) set to 0.49 ppm. The ordinate intensity is arbitrary units. Spectra were obtained under fully relaxed conditions using a 90° pulse and a 15-s recycle delay. The averaged FIDs were filtered at 20 Hz before Fourier transformation. The spectrum of the bladder is the result of 40 scans; the spectrum of the uterus is the result of 120 scans. The temperature of each preparation was 23 °C. The identities of the peaks is given in the text.
Values are expressed as parts/million ± 1 S.E. of the mean. Preparations were unstimulated and at 24–26 °C, except for hearts, which were studied while beating, at 37 °C. "—" indicates that no peaks were measurable. Biceps data were taken from Ref. 16; rat heart data are from S. Buchthal and T. R. Brown, unpublished observations, at 162 MHz.

<table>
<thead>
<tr>
<th>Peak no. (major chemical component)</th>
<th>Bladder</th>
<th>Uterus</th>
<th>Biceps</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (PTEtn)</td>
<td>4.26 ± 0.01</td>
<td>4.29 ± 0.02</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2 (P)</td>
<td>2.46 ± 0.03</td>
<td>2.37 ± 0.04</td>
<td>2.36 ± 0.01</td>
<td>—</td>
</tr>
<tr>
<td>3 (GroPCho)</td>
<td>(0.49)*</td>
<td>(0.49)*</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4 (PCr)</td>
<td>-2.51 ± 0.01</td>
<td>-2.52 ± 0.02</td>
<td>(-2.52)*</td>
<td>(-2.52)*</td>
</tr>
<tr>
<td>5 (βATP)</td>
<td>-5.07 ± 0.01</td>
<td>-5.05 ± 0.05</td>
<td>-4.99 ± 0.004</td>
<td>-4.95 ± 0.01</td>
</tr>
<tr>
<td>6 (αATP)</td>
<td>-10.07 ± 0.01</td>
<td>-10.09 ± 0.04</td>
<td>-9.98 ± 0.004</td>
<td>-10.05 ± 0.006</td>
</tr>
<tr>
<td>7 (βATP)</td>
<td>-18.84 ± 0.03</td>
<td>-18.90 ± 0.08</td>
<td>-18.53 ± 0.02</td>
<td>-18.55 ± 0.01</td>
</tr>
<tr>
<td>N</td>
<td>10</td>
<td>4</td>
<td>7</td>
<td>10</td>
</tr>
</tbody>
</table>

* The chemical shift reference was PCr for the biceps and heart and GroPCho for the uterus and bladder.

### Table IV

**T1 values of spectral peaks**

Values are expressed in units of time (s) ± S.E.

<table>
<thead>
<tr>
<th>Peak no. (major chemical component)</th>
<th>Bladder</th>
<th>Uterus</th>
<th>Ischemic bladder</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (PTEtn)</td>
<td>2.8 ± 0.2</td>
<td>5.2</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>2 (P)</td>
<td>3.6 ± 0.5</td>
<td>3.1</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>3 (GroPCho)</td>
<td>3.3 ± 0.6</td>
<td>5.4</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>4 (PCr)</td>
<td>2.9 ± 0.1</td>
<td>3.3</td>
<td>—</td>
</tr>
<tr>
<td>5 (βATP)</td>
<td>1.0 ± 0.1</td>
<td>0.7</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>6 (αATP)</td>
<td>1.0 ± 0.1</td>
<td>1.2</td>
<td>—</td>
</tr>
<tr>
<td>7 (βATP)</td>
<td>0.9 ± 0.1</td>
<td>0.8</td>
<td>—</td>
</tr>
<tr>
<td>N</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

* Peak areas were too small to be measured.

### Table V

**Metabolite measurements contained chemically in neutralized perchloric acid extracts of perfused bladders and uteri**

Quantities are expressed in units of μmol/g (w/w) ± S.E.M., n = 4 in columns 1 and 3; quantities in column 2 are millimolar in total intracellular water and were derived from the data in column 1 (see text). P, PCr, creatine, and Glc-6-P were assayed enzymatically, others by HPLC.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Bladder</th>
<th>Uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>0.97 ± 0.17</td>
<td>1.45</td>
</tr>
<tr>
<td>ADP</td>
<td>0.23 ± 0.05</td>
<td>0.34</td>
</tr>
<tr>
<td>PCr</td>
<td>1.64 ± 0.08</td>
<td>2.45</td>
</tr>
<tr>
<td>Free creatine</td>
<td>1.33 ± 0.06</td>
<td>1.99</td>
</tr>
<tr>
<td>PCr/total creatine</td>
<td>0.55 ± 0.02</td>
<td>0.51</td>
</tr>
<tr>
<td>GTP</td>
<td>0.12 ± 0.03</td>
<td>0.18</td>
</tr>
<tr>
<td>GDP</td>
<td>0.04 ± 0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>UTP</td>
<td>0.15 ± 0.03</td>
<td>0.22</td>
</tr>
<tr>
<td>UDP-Glc</td>
<td>0.26 ± 0.06</td>
<td>0.39</td>
</tr>
<tr>
<td>NAD</td>
<td>0.29 ± 0.05</td>
<td>0.43</td>
</tr>
<tr>
<td>Glc-6-P + Fru-6-P</td>
<td>0.07 ± 0.02</td>
<td>0.10</td>
</tr>
<tr>
<td>+ Glc-1-P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>2.02 ± 0.48</td>
<td>2.40</td>
</tr>
<tr>
<td>PTEtn</td>
<td>1.09 ± 0.07*</td>
<td>1.63</td>
</tr>
</tbody>
</table>

* These bladders were not perfused and were extracted in trifluoroacetate; n = 7.

This observation is documented and analyzed further on in "Results."

The spin-lattice relaxation time constants (T1) of the seven peaks are given in Table IV. The ATP peaks had the shortest T1 values, and PTEtn and P; the longest. No change in the T1 values was found when the bladders were stimulated maximally with carbachol and rendered globally ischemic by interrupting the flow of perfusate for 1 h.

**Chemical Identification of the Spectral Peaks** — The following criteria were used to identify the chemical compound(s) present in the peaks labeled in Fig. 1: (a) the correspondence between the observed chemical shifts in the perfused preparations and the known chemical shifts for pure compounds at the same pH; (b) quantitative analysis of specific metabolites in an aqueous extract of the bladders and uteri by specific and independent chemical, enzymatic, or chromatographic analyses; and (c) the persistence of a single spectral line of enhanced intensity upon addition of a standard to the extract at more than two pH values.

The primary component of peak 1 is PTEtn. When standards of PTEtn and other phosphate monoesters were added to extracts of bladders at each of three pH values in the range of 4–9, there was exact coincidence of the putative PTEtn peak only with the PTEtn standard. The other compounds tested were sn-glycero(1)phosphate, sn-glycero(2)phosphate, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate, ribose 5-phosphate, phosphocholine, and phosphoserine. Spectral examination of the extracts at several pH values or with complete titrations about the pK values of the compounds was necessary to exclude these compounds as components of peak 1. PTEtn was also isolated and identified, and its tissue content quantified by HPLC. Furthermore, none of the glycolytic hexose phosphates, which were measured by quantitative enzymatic assays, were present in sufficient quantities (Table V) to account for a substantial fraction of peak 1. The high-resolution spectrum of the aqueous extract of bladder (Fig. 2) shows that a number of other unidentified compounds (upper left-most trace) are present at low concentrations in the monophosphate region. These increase the total spectral intensity of peak 1 beyond that accounted for by the PTEtn chemical analysis. For example, phosphocholine is the other prominent component of the many phosphomonooester peaks shown in the inset of Fig. 2, but it is not resolved in the tissue spectra.

Peak 2 was identified as P; peak 3 as GroPCho, and peak 4 as PCr by the same three criteria given above. The high-resolution spectra show the presence of a small amount of sn-glycero(3)phosphothanolamine, but this compound was not

and uterus correspond closely with each other (Table III) and with those given previously for smooth muscle tissues (7, 9). The spectral positions from the cat biceps, a fast-twitch skeletal muscle, and from the rat myocardium are also given in Table III. Smooth muscles have significant peaks corresponding to PTEtn and GroPCho that are not found in the cat biceps or rat myocardium. In both the bladder and uterus, the β-ATP peak was shifted significantly upfield by ~0.3 ppm with respect to what was observed in the striated muscles.
resolved in the spectra of the bladders or uteri (compare Figs. 1 and 2).

Peaks 5, 6, and 7 are composed primarily of the \( \gamma \), \( \alpha \), and \( \beta \) peaks of ATP, with contributions from UTP and GTP. These nucleotides, plus ADP and GDP, were identified in extracts of bladders and uteri by HPLC. The sum of the amounts of UTP and GTP present accounted for 22\% of the integrated intensities of peaks 5 and 7, as estimated from the chemical contents given in Table V. The central peak of the triplet of the \( \beta \)-UTP peak can be discerned in the high-resolution spectra of the bladder extract (Fig. 2) within the triplet of the \( \beta \)-ATP peak. Significant amounts of UDP-Glc were also identified in the spectra of extracts and by HPLC. This information, plus the addition of a UDP-Glc standard to the extract, identified the small peak at -12 ppm (between peaks 6 and 7) in the bladder and uterine spectra as UDP-Glc.

**TABLE VI**

Comparison of spectral areas and chemically measured content of rabbit bladder

<table>
<thead>
<tr>
<th>Peak and major component</th>
<th>Fractional peak intensity</th>
<th>Peak intensity/ NTP intensity</th>
<th>Chemical assay/ total NTP *</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEtn</td>
<td>0.164 ± 0.007</td>
<td>1.50 ± 0.15</td>
<td>0.88 ± 0.06</td>
</tr>
<tr>
<td>Pi</td>
<td>0.124 ± 0.010</td>
<td>1.20 ± 0.13</td>
<td>1.63 ± 0.13</td>
</tr>
<tr>
<td>GroPCho</td>
<td>0.132 ± 0.012</td>
<td>1.19 ± 0.10</td>
<td>1.63 ± 0.13</td>
</tr>
<tr>
<td>PCr</td>
<td>0.132 ± 0.013</td>
<td>2.30 ± 0.19</td>
<td>0.32 ± 0.16</td>
</tr>
<tr>
<td>NTP + NDP</td>
<td>0.132 ± 0.005</td>
<td>6.28 ± 0.11</td>
<td>0.22 ± 0.18 b</td>
</tr>
<tr>
<td>NTP + NAD + UDP-Glc</td>
<td>0.202 ± 0.023</td>
<td>2.03 ± 0.38</td>
<td>1.44 ± 0.23 c</td>
</tr>
<tr>
<td>NTP</td>
<td>0.107 ± 0.010</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Data from Table V, where NTP = ATP + GTP + UTP = 1.24 \( \text{mol/g} \).

**Quantitative Metabolite Analyses: Comparison of Chemical Assays with Spectral Intensities**—Chemical and enzymatic analyses of aqueous extracts of bladders and uteri were scaled to the wet weight of the tissue (Table V). Except for Pi, none of the compounds listed in the table were present in the extracellular space. In order to interpret the amounts measured chemically and those measured from the integrated intensities of the spectra in terms of the intracellular concentrations, a value for the extracellular volume was needed. Phenyl phosphonate, which has a chemical shift downfield from the metabolites of interest (Fig. 3), was used as an extracellular space marker. The serial spectra show the rapid appearance of phenyl phosphonate, which reached a steady state while the perfusate contained phenyl phosphonate, and the equally rapid washout upon the removal of phenyl phosphonate from the perfusate. The volume of extracellular fluid in the bladder was 0.33 ml/g, and was calculated from the phenyl phosphonate and ATP intensities, the concentration of phenyl phosphonate in the perfusate, and the chemically measured ATP content. We did not measure the extracellular volume in the uteri. The second column in Table V gives the metabolite content in concentration units in intracellular water.

The first column in Table VI represents the integrated
**FIG. 4.** $^{31}$P NMR spectra of an ischemic and stimulated rabbit bladder. **Bottom spectrum,** control, unstimulated conditions. **Next three spectra,** stimulation with 30 $\mu$M carbachol (CARB) added to the perfusate. The 8 uppermost spectra were obtained after making the stimulated bladder ischemic. NMR conditions are as in Fig. 3. The major components of several peaks are indicated.

**FIG. 5.** $^{31}$P NMR spectra of stimulated, ischemic, and reperfused bladder. **Bottom spectrum,** is from a bladder previously stimulated for 20 min with 30 $\mu$M carbachol. The major components of the peaks are identified. **Middle spectrum,** is of the same bladder after 110 min of ischemia while maintaining stimulation with carbachol. PCr and ATP have been depleted, resulting in a large Pi peak and residual NAD peak. **Top spectrum,** is from the same bladder after re-establishing perfusion for 30 min with 30 $\mu$M carbachol. NMR conditions are as in Fig. 3.

The intensities of the seven peaks labeled in Fig. 1 as fractions of the total $^{31}$P spectral intensity for spectra collected under fully relaxed NMR conditions (90° pulse, 15-s intervals). The quantities given in the second column of Table VI are the same data scaled to the integrated intensity of the total nucleotide triphosphate (NTP) peak (peak 7), which is composed predominantly of ATP, but contains UTP and GTP as already described. The data in the third column of Table VI is derived from chemical data given in Table V by scaling the chemical content to the sum of the chemically measured NTP, 1.24 ± 0.14 pmol/g, derived from the data in Table V. Thus, the data in the second and third columns of Table VI are directly comparable, and test whether the spectral analyses of the intact tissues agree quantitatively with the chemical analyses of our tissue extracts. The content of NTP is a useful quantity with which to normalize the chemical and spectral data because it is composed of ATP and other NTPs that are chemically stable during the preparation of the extracts, and because ATP content measured chemically and by spectral integration has been shown to agree quantitatively (35, 36).

Furthermore, PCr is in the cytosol and has the narrowest spectral line in our preparations. There is a close correspondence between the PCr:ATP ratio measured from the spectral intensities (column 2) and by chemical analyses (column 3). If there is a large amount of nucleotide bound so as to be invisible in the spectra, the quantitative data in column 2 should systematically exceed those in column 3, and this did not occur. Therefore, it is highly unlikely that there is a significant amount of nucleotide present in the tissues that is NMR-invisible but extractable by perchloric acid.

The chemically measured amount of PEtn accounts for one-half of the spectral intensity of peak 1. The rest of the intensity may be accounted for by the other phosphate monoester compounds detected in the spectra of the extract (Fig. 2). We did not identify or quantify all of those components. Note that less than 4% of peak 1 could be accounted for by the measured tissue content of the glycolytic hexose phosphates.

Although the value for the chemically measured Pi content tended to be larger than that derived from the spectral inten-
sity, the difference was not statistically significant. A peak of 
PCr in the bladder and uteri spectra was present throughout 
the entire course of our experiments, whereas in other reports 
(7, 10–12) the PCr peak was very small or undetectable. Of the 
total of 2 μmol/g (Table III) present in perfused bladder, 0.4 
μmol/g was calculated to be present in the extracellular vol-
ume (the perfusate contained 1.16 mM Pi). Thus, the intra-
cellular PCr concentration was estimated to be 2.4 mM (2 μmol/ 
g − 0.4 μmol/g in the extracellular volume, divided by 0.67, 
the intracellular volume fraction).

While there tended to be a difference between the chemi-
cally measured content of the components of peak 6 (com-
posed of the α peak of ATP and peaks of UDP-Glc and 
NAD+) and the spectral intensity, the difference was not 
statistically significant. The integrated intensity of the γ-
NTP (peak 5) statistically (p < 0.05) exceeded that of the 
NTP (peak 7), and the possible significance of this is discussed 
later.

Chemical Changes in Stimulated and Ischemic Bladders—
The goal of these experiments was to deplete the high-energy 
phosphate content in the perfused bladder and to identify, if 
possible, an ADP peak whose chemical shift would also reflect 
the extent of binding to Mg2+. The series of spectra in Fig. 4 
show that, after maximal carbachol stimulation for 30 min, 
the chemical changes were small and confined to a decrease 
in PCr, an increase in Pi, and a decrease in pH to 6.9. If the 
bladders were also made ischemic, the extent of the chemical 
changes were larger and occurred more rapidly. PCr content 
decreased to undetectable levels after 60 min of ischemic 
stimulation with carbachol, and Pi content increased. The 
NTP content decreased only after most of the PCr dis-
appeared. In the example shown in Fig. 4, the pH decreased 
after 70 min of ischemia to 6.4. These changes were reversible, 
as the spectra obtained in another experiment (Fig. 5) show.

A peak assigned to β-ADP could be resolved in 7 of 13 
bladders subjected to 40–90 min of maximal carbachol stim-
ulation plus total ischemia; one example is shown in Fig. 6. 
The observed chemical shift of β-ADP was −6.67 ± 0.10 ppm, 
and that of the β-ATP peak was −18.83 ± 0.06 ppm; the 
averaged pH was 6.6.

Estimates of the Free Intracellular Magnesium Ion Concentra-
tion—In order to estimate the intracellular free Mg2+ concen-
tration from the chemical shifts of β-ATP and β-ADP, titration 
curves for Mg2+ binding at pH 7.0 and 6.6 were made 
in vitro under conditions mimicking the cytoplasm, as 
described under “Experimental Procedures.” The pH of 6.6 was 
chosen because it was the average intracellular pH observed 
in the 7 bladders in which an ADP peak was identified. From 
the titration curves (Table II and Fig. 7), the estimated free 
Mg2+ concentration could be determined from the observed 
chemical shifts of the β peak of ATP at pH 7.0 from normally 
perfused bladders (Fig. 7A) and from the chemical shifts of 
β-ATP and β-ADP at pH 6.6 from ischemic and stimulated 
bladders (Fig. 7B). From these data we conclude that the 
normally perfused and unstimulated bladder had a free Mg2+ 
intracellular concentration of 0.46 ± 0.03 mM; the correspond-
ing value for the uterus was 0.40 ± 0.07 mM. In the stimulated 
bladder, the free Mg2+ concentration was 0.50 ± 0.09 
mM and 0.45 ± 0.10 mM, as estimated from the ATP and 
ADP chemical shift data, respectively. Using the same cali-
bration procedure, we derived an intracellular Mg2+ concen-
tration in fast-twitch skeletal muscle (cat biceps) of 1.5 ± 0.3 
mM, and for cardiac muscle (rat myocardium) a value of 1.4 ± 0.1 mM. The total Mg2+ content of rabbit bladders as 
measured by atomic absorption analyses was 6.6 ± 0.6 (n = 
4) μmol/g.

DISCUSSION

Chemical Composition—The presence of PEtn as a quantita-
tively significant component (1 μmol/g) of smooth muscle is 
not widely recognized, although its presence in a large 
number of tissues has long been known (37, 38). In our work 
the identity of PEtn was based on a number of criteria, 
including the HPLC separation of PEtn from extracts of the 
bladder. Sugar phosphates as a generic class of compounds 
were barely detected by specific enzymatic analyses in well-
perfused and unstimulated muscles. We have excluded the 
presence of significant amounts of a large group of other 
phosphomonoesters in our preparations, as indicated in the
second section of the "Results." Therefore, a number of papers (7-13) probably have incorrectly assigned this prominent peak in smooth muscle \(^{31}\)P NMR spectra as a component of carbohydrate metabolism, rather than as PEtn, which is a phospholipid metabolite. Because of variation in the handling of the preparations in which there may have been a stimulation of glycolysis, we cannot state with certainty that previous assignments of peaks present in the monophosphate ester region as sugar phosphates (7-13) are incorrect. However, we do believe that those experimental conclusions are suspect and should be re-examined. GroPCho was also a prominent and previously unrecognized component of smooth muscle (0.8 \(\mu\)mol/g). PEtn and GroPCho are phospholipid metabolites, whose function in smooth muscle is unknown. Substantial concentrations of these compounds, especially PEtn, tend to be found in proliferating and secretory tissues (37-39), in immature brain (40-42), and in tumors (43-45). We therefore speculate that smooth muscle may have a high phospholipid turnover rate, possibly associated with an unexpected synthetic, secretory, or transport function. GroPCho is present in many rabbit tissues, and Burt et al. (46, 47) have suggested roles for this compound in membrane processes. Clearly, information on the role of these compounds in the function of smooth muscle would be desirable.

Another observation is the presence of significant amounts of UTP and GTP in bladder and uterine smooth muscle. The presence of UTP and GTP in bovine arteries (49) and in rat uterus (50) has been reported, but more recent investigations of chemical changes in smooth muscle do not appear to have distinguished adenosine from other NTPs (14, 15). As indicated by the data in Table V, ATP comprises only 78% of the total NTP content. Thus, PCr by the creatine kinase reaction is buffering only part of the NTP pool during contractile activity. No significant amounts of cytidine nucleotides were detected in the HPLC chromatograms. This absence is noteworthy in light of the speculation on phospholipid turnover in smooth muscle, because CDP derivatives are intermediates in phospholipid biosynthesis.

The data presented in Table VI indicate that the spectral intensity of \(\gamma\)-NTP (primarily the \(\gamma\) peak of ATP) was significantly less than that of the \(\beta\) peak. The \(\beta\) peak occurs only in NTP, whereas the \(\gamma\) or terminal peak occurs in both the tri- and diphosphate forms. This NMR result thus provides some evidence for an NMR-detectable ADP content in \(^{31}\)P NMR spectra of normal, unstimulated bladders. Opposing this interpretation is the result that the ADP + GDP content estimated from the spectral intensities of bladder (0.35 ± 0.14 \(\mu\)mol/g, derived from column 2 of Table VI) is not different from the ADP + GDP content measured in HPLC extracts (0.27 ± 0.05 \(\mu\)mol/g from Table V). We conclude that the apparent quantitative difference between the \(\gamma\) and \(\beta\) peaks is an artifact of the unstimulated bladder, and so we cannot establish the presence of ADP in the spectra of normal bladder.

We consistently observed a \(P_i\) peak, and found in well-perfused unstimulated bladders a tissue content of 2 \(\mu\)mol/g. This observation of a persistent \(P_i\) peak throughout the course of our experiments is at odds with other reports on smooth muscle preparations (7, 10-12), where the absence of a \(P_i\) peak was considered to represent the normal physiological state. There are two important differences between our preparations and those of other investigators: (a) in our experiments, the bladder was perfused through its natural arterial and capillary supply, rather than being supplied by diffusion from a superfusate; and (b) the perfusate, and thus the extracellular fluid, in our experiments contained 1.2 mM \(P_i\), whereas in most other experiments, no phosphate was added (most likely because its presence in the large volume of superfusate would have obscured the tissue spectral peaks). It is thus possible that under certain circumstances \(P_i\) was leached from smooth muscle strips when no \(P_i\) was present in the surrounding medium. A second possible explanation for the reported observation of low or absent \(P_i\) peak in smooth muscles studied; PCr in striated muscles was set as an internal reference to -2.52 ppm, whereas the internal reference used in the smooth muscles was GroPCho at 0.49 ppm. Thus, there is no evidence for systematic differences in the magnetic susceptibility between smooth and striated muscles. Cohn and Hughes (17) showed that chemical shifts of the phosphate groups in nucleotides can be caused by the binding of divalent cations other than Mg\(^{2+}\), but it is clear that in resting smooth muscle as well as in other muscles, the predominant intracellular divalent cation is Mg\(^{2+}\) (52, 53). We have taken temperature and pH into account in our calibrations and have accurately measured the intracellular pH in those bladders in which the nucleotide chemical shift data are also measured.

The evidence that the intracellular calcium concentration is similar in all types of muscle cells is that the ionic composition is also similar (48). These arguments lead us to conclude that the chemical shift of nucleotide peaks (predominantly ATP, and in ischemic preparations of ADP as well) can be used to provide meaningful estimates of the cytosolic free Mg\(^{2+}\) content, as has been done for other tissues and cells (16, 18-21).

The simplest interpretation of the significant upfield shift of the \(\beta\) peak of ATP in bladder and uterine is that the \(P_i\) peak was less saturated by Mg\(^{2+}\) in the smooth muscles. The fractional saturation can be obtained from these data and the in vitro calibration curve by the use of formulae like those given by Gupta and Moore (19). For the striated muscles (fast-twitch cat biceps and rat myocardium), the fractional content of total NTP not complexed with Mg\(^{2+}\) was 0.05; the same fractions for uterus and bladder were, respectively, 0.17 and 0.15. A similar fraction of uncomplexed nucleotide was reported by Degani et al. (12) in rat uterus, but in other reports of smooth muscle \(^{31}\)P NMR studies no quantitative conclusions are reached.

Quantitative values for the free Mg\(^{2+}\) concentration were obtained from the measured chemical shifts with the appropriate calibration curves (Table II and Fig. 7). Our results show that the free Mg\(^{2+}\) concentration in smooth muscles (0.5 mM) is about one-third of that in striated muscles (1.5 mM). There may be some error in assigning a value for the free Mg\(^{2+}\) concentration in the calibration solutions, because the
composition of the solution was determined by a multi-equation calculation, and therefore depends on the accuracy of all the association constants used (Table I). Our confidence in the accuracy of our calibration method is based on the fact that we used the same calculation to predict the pH of the final solution, and we found the predicted and measured pH to agree within 0.02 units. Recall that not all of the $\beta$-ATP signal is derived from ATP, but only 78% of it. The balance is derived from UTP and GTP, but their proton and magnesium ion association constants for comparable metal-ligand reactions are within 2% of the values for ATP (54). Furthermore, the chemical shifts of the $\beta$ peaks of ATP and ribose 5'-triphosphate as a function of $\text{Mg}^{2+}$ binding were the same (55). We thus conclude that there is little error in our quantitative analysis due to the heterogeneous chemical composition of peak 7.

The $\beta$ peak of the dimagnesium complex (Mg$_2$ATP) had a chemical shift upfield of the shift of MgATP (55, 56). Thus, the upfield shift of $\beta$-ATP that we observed in smooth muscle spectra compared to that of striated muscle could have an ambiguous interpretation, since both the dimagnesium complex and the uncomplexed ATP are shifted upfield with respect to MgATP. Because the association constant of the Mg$_2$ATP complex was 30-40 M$^{-1}$ (55, 57), the hypothesis that a significant amount of the total ATP was in the form of Mg$_2$ATP in the bladder and uterus requires the free $\text{Mg}^{2+}$ to be in molar excess over ATP. An estimate of the required $\text{Mg}^{2+}$ content can be obtained using the formula given in Table II and the chemical shifts for MgATP$^-$ and Mg$_2$ATP given by Bishop et al. (55). The result is that the free $\text{Mg}^{2+}$ concentration needs to be approximately 25 mM to satisfy the hypothesis that there is a significant content of the dimagnesium complex. This concentration is more than 3 times the total $\text{Mg}^{2+}$ content of the bladders, which we measured to be 6.6 $\mu$mol/g. Thus, the hypothesis that the upfield shift of $\beta$-NTP observed in smooth muscles compared to striated muscles is due to the presence of Mg$_2$ATP is excluded.

The estimated intracellular free $\text{Mg}^{2+}$ concentration represents a weighted average throughout the cell and among all of the cells. The simplest interpretation, subject to the limitations discussed below, is that the measurements of intracellular pH and $\text{Mg}^{2+}$ that we and others measure by NMR methods reflect predominantly the properties of the cytosol. It should be noted that in our experiments only single resonances were observed for all of the peaks, as reported for other tissues, including erythrocytes, which contain no intracellular organelles (18). This behavior was expected for an association reaction in rapid exchange in a homogeneous solution (1-3, 17), and was observed for the cases of proton exchange with P, and of $\text{Mg}^{2+}$ exchange with the nucleotides. However, there is clearly the possibility for intracellular metabolite compartmentalization in a number of intracellular organelles (58), e.g. in the mitochondrial matrix. The mitochondrial volume fraction in vascular smooth muscle is on the order of 5-10% of total cell volume (58). In rat urinary bladder it is estimated that the mitochondrial volume fraction is somewhat lower (2-4%). Mitochondrial matrix ATP appears fully complexed with $\text{Mg}^{2+}$ (59). Thus, to the extent that there is a significant mitochondrial ATP pool, the estimated cytosolic $\text{Mg}^{2+}$ concentration would be lower than the overall average value reported here.

The apparent dissociation constants for MgATP and MgADP under conditions mimicking the cytoplasm can be directly derived from the pK' data given in Table II. Those constants are mixed dissociation constants which take into account all of the metals bound to all of the ligands listed in Table I, according to the mole fractions of the various complexed species present in the solution. Note that the number of interacting ionic species used in our calibrations is considerably larger than that used by others (19-21, 60, 61). The apparent dissociation constants so derived are: 78 $\mu$M for MgATP at pH 7, 91 $\mu$M for MgATP at pH 6.6, and 1.0 mM for MgADP at pH 6.6. These apparent constants are 2-fold larger than those used by Gupta and colleagues (19, 20) and by Garfinkel and Garfinkel (60). Our estimate of the free $\text{Mg}^{2+}$ concentration is therefore about twice that calculated for frog skeletal muscle by Gupta and Moore (19), but similar to the value given by Garfinkel and Garfinkel (60) in their recalculation of the data of Wu et al. (61), and is within the wide range of values given by ion-selective microelectrodes (62) and by indicator dye absorption (63). However, there is no necessity that all of the muscles so far studied have the same concentration of free $\text{Mg}^{2+}$ in the cytosol, especially since comparisons have been made between homeothermic animal tissues and those of poikilothermic ones, and among the different types of cells. Our results indicate that the cytosolic $\text{Mg}^{2+}$ concentration in rabbit smooth muscle is 0.5 mM, which is similar to the free $\text{Mg}^{2+}$ concentrations found in erythrocytes (18, 64, 65) and lymphocytes (66), but about 3-fold lower than found in striated muscles.

The implications of a submillimolar intracellular $\text{Mg}^{2+}$ concentration for cellular function are many. In erythrocytes, the Na/K pump activity was influenced by $\text{Mg}^{2+}$ concentration (65). Hypertensive patients have lower concentrations of free $\text{Mg}^{2+}$ in their erythrocytes (20). For smooth muscle cells, it can readily be calculated that if intracellular $\text{Mg}^{2+}$ were in equilibrium with extracellular $\text{Mg}^{2+}$, and if the membrane potential was $-60$ mV, the ratio of $[\text{Mg}^{2+}]_{i}/[\text{Mg}^{2+}]_{o}$ would be about 100. Assuming the cell membrane has a finite permeability to $\text{Mg}^{2+}$, there must therefore be an outwardly directed transport mechanism to maintain intracellular $\text{Mg}^{2+}$ as low as our measurements indicate. The Ca$^{2+}$-activated ATPase activity of actomyosin from smooth muscle (22) and the velocity of shortening in glycinated smooth muscle strips (23-25) was a function of the $\text{Mg}^{2+}$ in the millimolar and lower range of concentration. Increase in the free $\text{Mg}^{2+}$ concentration in rat uterus by estrogen treatment (12) has been reported. It will be important to look for additional examples of changes in the intracellular $\text{Mg}^{2+}$ concentration in smooth muscle cells under a variety of functional states to see whether free $\text{Mg}^{2+}$ concentration is a physiological variable, and what mechanisms might be regulated by cytosolic $\text{Mg}^{2+}$ concentration.

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