Vol. 197, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1993 Pages 75-84

DETECTION OF PHOSPHODIESTER RESONANCES IN THE PERFUSED HEART FROM VERTEBRATE ECTOTHERMS WITH NUCLEAR MAGNETIC RESONANCE

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Received September 28, 1993

Summary: *In vivo* ^{31}P NMR has been used to characterize the phosphorylated compounds present in the heart from vertebrate ectotherms. The perfused hearts from all animals experimented showed prominent resonances between the inorganic phosphate and phosphocreatine peaks. The pattern of these compounds was found to be different in the heart of the different species. As shown by ^{31}P and proton NMR of perchloric extracts, the chemical shift of some of the compounds was characteristic of glycerophosphorylcholine, glycerophosphorylinositol, phosphorylcholine, phosphorylserine, phosphorylethanolamine and phosphoenolpyruvate. The non-identified resonances were found to be phosphodiesters, as demonstrated by alkaline phosphatase hydrolysis. The physiological significance of these high levels of phosphodiesters in the heart from vertebrate ectotherms is discussed.

In vivo ^{31}P NMR is of great use for assessing the levels of high-energy phosphorylated compounds and for determining the intracellular pH in the heart from living animals (1, 2). Yet, although the cardiac metabolism has been extensively analysed in mammals (3-10), only one study in heart from an ectotherm vertebrate, the turtle, had been carried out up to now by this technique (11).

In all species, spectra from isolated perfused heart show prominent resonances corresponding to phosphocreatine and ATP, and minor resonances from inorganic phosphate and phosphomonoesters. Spectra from some mammals (rabbit, ferret, human) and from turtle

0006-291X/93 $4.00

display one additional resonance between the inorganic phosphate and phosphocreatine peaks (8-10), which has been assumed to be a phosphodiester resonance (8, 10, 15). ^{31}P NMR experiments (8, 15) have shown that the phosphodiester content did not vary significantly during ischemia or anoxia. Up to now, the physiological role of such high amounts of phosphodiesters in the myocardium of some species is unknown.

Based on the observation of ^{31}P and ^{1}H NMR signals in the heart from several vertebrate ectotherm distinct species: fish (rainbow trout, european eel, dogfish, sea bream, skate, turbot and sea-bass) and amphibia (frog), the occurence of prominent resonances in the phosphodiester region in all spectra is discussed.

Materials and Methods

Animals: Trouts (*Onchorhynchus mykiss*) and eels (*Anguilla anguilla*) were obtained from local dealers. Dogfish *(Scyliorhinus canicula)* and skate (*Raja clavata)*were caught in the Bay of Concarneau. Farmed sea bream *(Spondyliosoma cantharus)*, sea bass (*Dicentrarchus labrax)*, turbot *(Psetta maxima)* were supplied by IFREMER (Brest, France). Frog (*Rana esculenta)* and rat were supplied by the Centre Animalier d'Ardennais (France).

Heart preparation: Rats were anaesthetized by ether inhalation. The heart was rapidly dissected and perfused using the classic Langendorf method.Fish were anaesthetized by immersion in ethyl carbamate solution (30 g/l) till ventilation stopped. Frogs were pithed. The heart was rapidly dissected and two cannulae inserted and secured : one in the hepatic vein and the other in the ventral aorta. All the other veins were ligatured. Heparin containing medium was injected via the hepatic vein to avoid blood cloting. An hydrostatic and dynamic postcharge was achieved by a 10 cm capillary tube connected to the ventral aorta. In these conditions, the heart was spontaneously beating during a period largely exceeding the duration of one experiment.

The heart was connected to the perfusion apparatus and placed in a 10 mm diameter NMR tube.The saline was delivered to the heart by a peristaltic pump at a constant flow (from 1.70 to 3.40 ml/min for all fish (except eel) and frog heart and 6.70 ml/min for rat and eel heart) from a 500 ml reservoir, another pump being used to remove the saline from the tube.

The different perfusion media correspond to the plasmatic concentration and pH of fish (12), to a modified Ringer solution (frog), and to a Tyrode solution (rat).The medium was gassed with bubled oxygen and maintained at 15 $\pm$ 1°C for ectotherm heart and 23 $\pm$ 1°C for rat heart.

NMR experiment: NMR spectra were collected on a 7.04 T Bruker AC300 NMR spectrometer using a 10 mm variable temperature probe. Probe temperature was maintained at 15$\pm$1°C exept for rat heart (23 $\pm$ 0.3°C).

For the *in vivo* experiments, phosphorus spectra were acquired at 121.5 MHz for 12 min (256 pulses) using the following acquisition parameters: recycle delay 2 sec, pulse angle 30° (7 μs), spectral width 8064.516 Hz, and data table size 32 K. The total recycle time between pulses was 3 sec.The phosphometabolite absolute concentrations were calculated from the knowledge of the ATP level measured after biochemical extraction and H.P.L.C. analysis, assuming that all ATP is visible in NMR spectra (11).

^{31}P *in vivo* spectra were compared to spectra of perchloric acid extracts (see "Biochemical analysis") produced in parallel, from freeze clamped heart just after having recorded *in vivo* spectra. After neutralization at the physiological pH (7.2), the extracts were checked by ^{31}P NMR for identification of acid-soluble-phosphorus containing metabolites, after changing the pH and adding known compounds to the solution. The phosphocreatine peak was used as an internal standart at 0 ppm. The chemical shift of phosphocreatine was referenced to external 85% H_3PO_4. Before analysis, the samples were dissolved in 50 mM imidazole buffer, pH 7.1, containing final concentrations of 20.3 mM Na^+, 129 mM K^+, 88.6 mM Cl^- and 9.3 mM Mg^{2+}. The ionic content of this buffer represents the average ionic environment of fish heart cells (12). High resolved spectra were obtained by this method. Alcaline phosphatase (10 U) was added to trout heart perchloric extracts and to a mixture of 2mM glycerophosphorylcholine, 2mM ATP and 2 mM phosphocreatine as control. ^{31}P NMR spectra were recorded before addition and after 10 min.
Additionally, the extracts were checked by proton NMR for further identification of acid-soluble containing metabolites. Before analysis, the lyophilized extracts were dessicated and dissolved in D_2O (99.9%). The definitive attributions were made after running a series of spectra obtained after addition of known compounds to the perchloric acid extracts. The ^1H resonance due to the methyl group of Cr occurs at approximatively 3.4 ppm relative to the H_2O signal at 4.82 ppm. The interpulse delay was 2.7 μsec.and the flip angle: around 8° Proton spectra were acquired by summing 32 FIDs. Chemical shifts are shown as ppm relative to the $C_6H_9D_4NaO_2Si$ signal.

Biochemical analysis: In order to calculate the absolute concentrations from *in vivo* ^{31}P NMR spectra, biochemical extracts were performed and the ATP concentration measured by HPLC. The biochemical extraction was performed as follows: at the end of the NMR experiment, hearts were freeze-clamped in liquid nitrogen and then homogenized under liquid nitrogen in six volumes of 0.6 N perchloric acid. Homogenates were centrifuged at 31 000 x g for 15 min at 4°C and the supernatants were neutralized with 5 N K_2CO_3. After centrifugation at 31 000 x g for 15 min. at 4°C, the samples were lyophilized and stored at -80°C.The H.P.L.C. analysis was performed as previously described (13).

Results and Discussion

Fig. 1 shows representative ^{31}P NMR spectra obtained during perfusion in the heart of different ectotherm species, compared to rat. Our experimental spectrometric conditions allowed us to obtain a high signal to noise ratio, and a satisfying precision leading to distinguish several phosphodiester resonances.

The NMR spectra obtained from the perfused hearts in the all ectotherm species exhibited different major peak positions at resonances ranging from 3.6 to 1.6 ppm and occasionally, a minor one around 6.8 ppm (Table 1). These peaks were not observed in the ^{31}P NMR spectra from rat heart treated under similar conditions (Fig.1).

The high energy phosphometabolite concentrations, calculated from the knowledge of the absolute ATP concentrations, were found to be much higher in rat than in fish (Table 2). When fish were compared, higher levels of the high energy phosphate compounds:

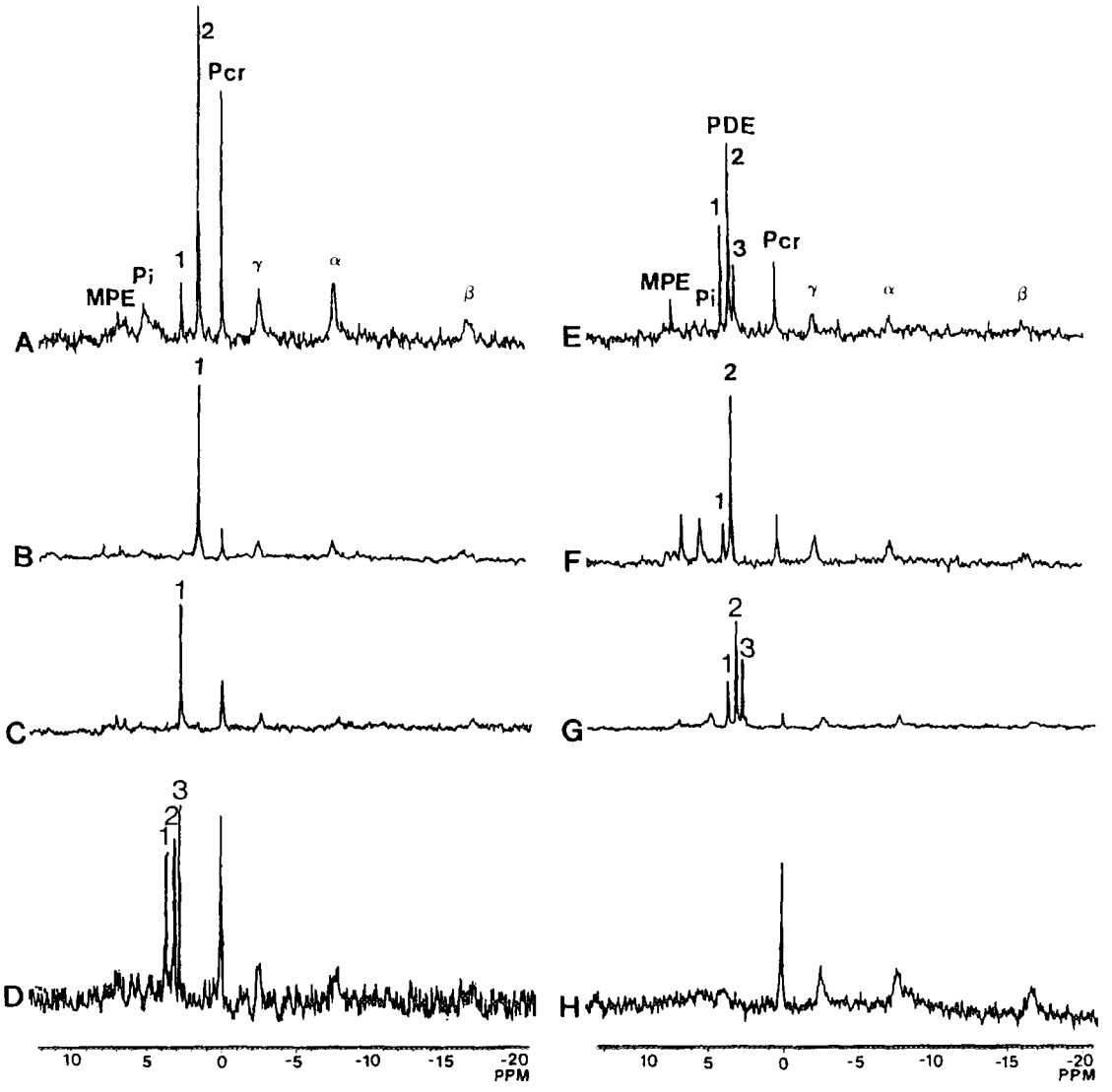

Figure 1. ^{31}**P NMR spectra of perfused heart.**

Panel A: trout. Panel B: sea bream. Panel C: turbot. Panel D: frog. Panel E: eel. Panel F: dogfish. Panel G: skate. Panel H: rat.
Spectra were acquired under the following conditions: 256 acquisitions, pulse angle 30°, interpulse delay 3 sec. Zero ppm reference: Phosphocreatine.
MPE: monophosphate esters; Pi: inorganic phosphate; PDE: phosphodiesters; Pcr: phosphocreatine; γ, α, β: phosphorus atoms of ATP.

phosphocreatine, ATP and phosphomonoesters were observed in trout heart than in eel heart (Table 2), the phosphodiester total concentration in trout and eel hearts corresponding to 27 and 48% of the total phosphometabolites respectively.The different absolute levels of the high

Table 1: Identification of resonances in perfused heart from some ectotherms

Assignment	Chemical shift (ppm)						
	trout	eel	dogfish	sea bream	skate	turbot	frog
MPE and PE	6.80±0.15	6.86±0.02	6.79±0.04	6.89±0.08			
PC			6.25±0.03				
Pi	5.17±0.06	5.16±0.10	5.08±0.02	5.33±0.05			
Unknown compound		3.57±0.02	3.58±0.01		3.57±0.02		3.59±0.02
GPI and GPC	2.68±0.02	3.03±0.02	3.04±0.01		3.03±0.01	2.68±0.01	2.71±0.55
Unknown compound		2.60±0.02					2.59±0.13
Unknown compound	1.56±0.01			1.57 ±0.01			

The intracellular pH was around 7.2.The chemical shifts are expressed relative to phosphocreatine. Values represent the means ± SD (n = 4).

energy phosphates in the heart of trout and eel indicate some differences in the energetic events in these species, probably related with their locomotor activity, the more active fish, trout, displaying higher concentrations of phosphocreatine, compared to the ATP concentration, as shown by the ATP/Pcr ratio (Table 2). The higher phosphometabolite levels found in the rat myocardium are related to the more compact structure of this tissue, the fish myocardium being partially spongious (14).

Table 2: Phosphometabolite content in isolated, perfused hearts

	trout	eel	rat
ATP	8.375 ± 2.767	5.249 ± 2.236	24.323 ± 2.542
Pi	8.417 ± 7.813	4.786 ± 2.432	18.045 ± 1.121
Pcr	17.928 ± 8.150	6.722 ± 1.472	43.428 ± 3.357
P.M.E.	11.284 ± 7.867	9.599 ± 3.275	10.321 ± 5.262
$P.D.E._1$	-	6.873 ± 2.687	0
$P.D.E._2$	-	10.068 ± 3.261	0
$P.D.E._3$	3.721 ± 2.962	7.952 ± 4.493	0
$P.D.E._4$	13.471 ± 4.364	-	0

The absolute levels of phosphomonoesters(P.M.E.), phosphodiesters (P.D.E.), inorganic phosphate (Pi) and phosphocreatine (Pcr), calculated from ^{31}P NMR data and HPLC analysis of ATP are expressed in μmol/g wet weight.Values are means ± S.D.

To further characterize the compounds corresponding to the unknown resonances detected in the *in vivo* spectra, NMR studies were also made on perchloric extracts from the corresponding cardiac tissues. The unknown resonances detected in the *in vivo* spectra are also observed in the ^{31}P NMR spectra obtained from perchloric extracts, excluding the fact that these compounds are phospholipids (Fig. 2). The non identified resonances are phosphodiesters, as shown by alkaline phosphatase hydrolysis (Fig.3).

The phosphomonoesters peak, around 6.8 ppm, consisted of fructose-6-phosphate, fructose-1,6-diphosphate, AMP, IMP, phosphorylserine and phosphorylethanolamine. The contribution of these two last compounds to the area of the phosphomonoesters peak seemed to be higher in the perfused eel and sea bream hearts. The glucose-1-phosphate resonance arose near 5.4 ppm,

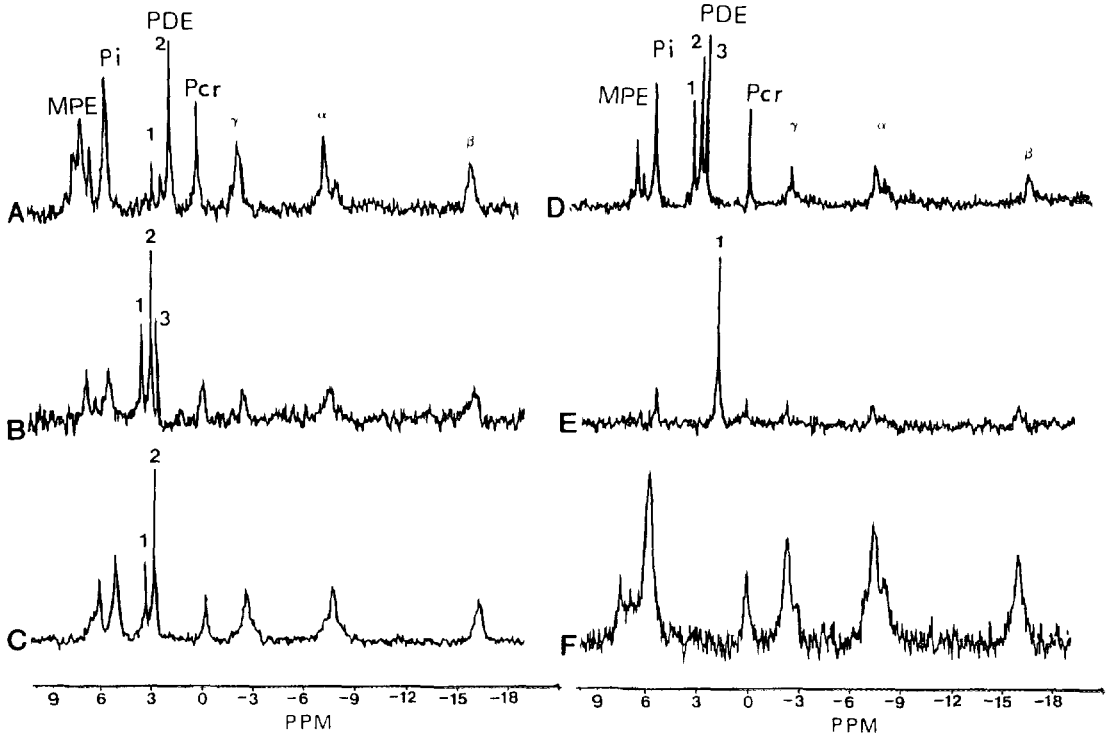

Figure 2. ^{31}P NMR spectra of perchloric acid extracts of heart.

Panel A: trout. Panel B: eel. Panel C: dogfish. Panel D: frog. Panel E: sea bream. Panel F: rat.
Spectra were acquired under the following conditions: 512 acquisitions, pulse angle 30°, *interpulse* delay 3 sec. *Zero ppm reference*: Phosphocreatine.
MPE: monophosphate esters; Pi: inorganic phosphate; PDE: phosphodiesters; Pcr: phosphocreatine; γ, α, β: phosphorus atoms of ATP.

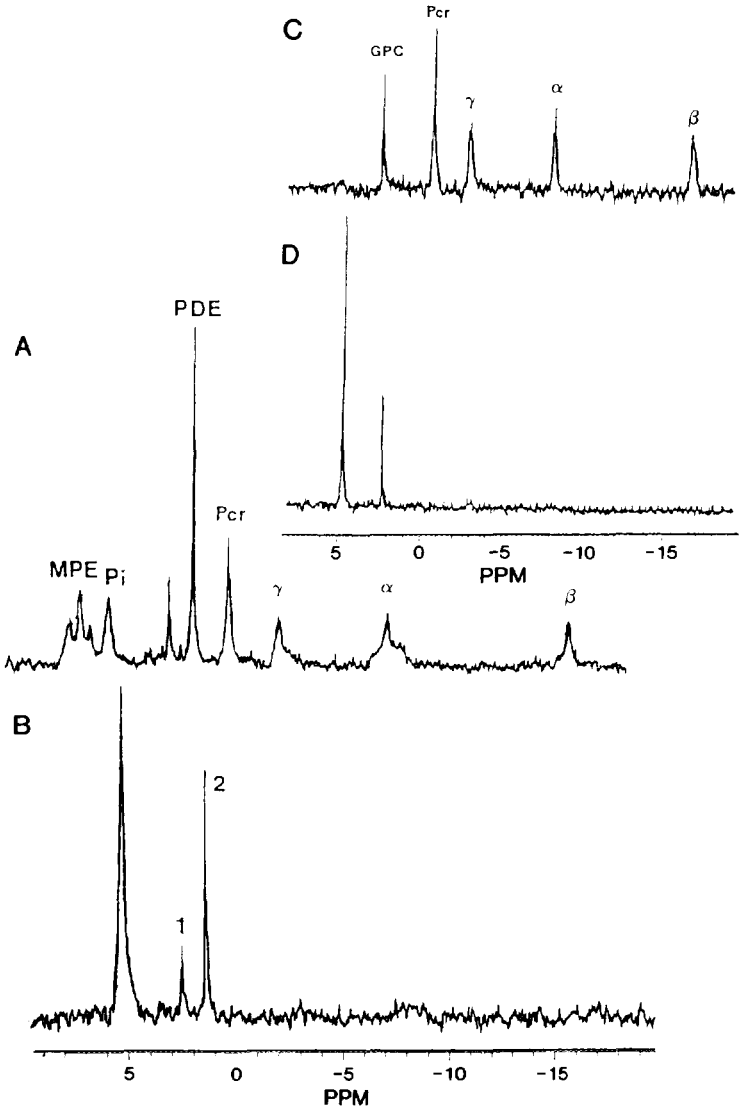

Figure 3. Effect of alkaline phosphatase on ^{31}P NMR spectra of perchloric acid extracts of trout heart (A) and of a solution of phosphorylated compounds (B).

A: trout heart perchloric extract.
B: Alcaline phosphatase (20 U) was added a to trout heart perchloric extract.
C: mixture of 2mM glycerophosphorylcholine, 2mM ATP and 2 mM phosphocreatine.
D: Alcaline phosphatase (10 U) was added to a mixture of 2mM glycerophosphorylcholine, 2mM ATP and 2 mM phosphocreatine as control.
^{31}P NMR spectra were recorded before addition and after 10 min.

very close to that of inorganic phosphate. Finally, the 3.57-3.59 ppm resonance was found in the eel, dogfish, skate and frog hearts and was not identified. The 2.68-3.04 ppm resonance, found in the trout, eel, dogfish, skate, turbot and frog hearts, corresponded to the

glycerophosphorylinositol-glycerophosphorylcholine resonance. The resonance around 2.60 ppm was found in the eel and frog hearts and was not identified. The 1.56 ppm resonance was specific of trout and sea bream hearts and was not identified.

Fig. 4 shows representative proton spectra obtained from perchloric extracts from trout, eel and frog hearts. As shown in ^{31}P NMR spectra, one of the major peaks (trout and eel) and

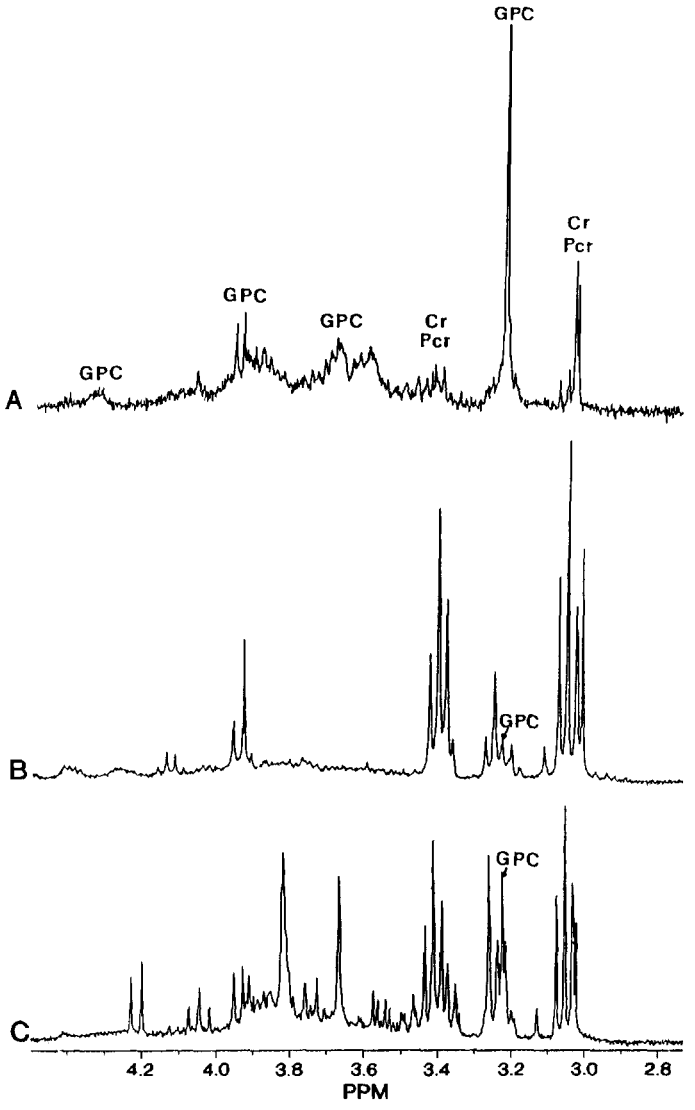

Figure 4. Proton NMR spectra of perchloric acid extracts of heart.

Panel A: frog. Panel B: trout. Panel C: eel.
Spectra were acquired under the following conditions: pulse width: 1 μsec., acquisition time: 2.7 sec., sweep width: 6024 MHz, digitalized into 32 K data points, and the sum of 32 free induction decays (FIDs). Zero ppm reference: $C_6H_9D_4NaO_2Si$.

the major peak, corresponded to glycerophosphorylcholine. A few amount of this compound was present in trout. The other unknown compounds could be derivated from choline.

Of interest, is the finding of phosphodiesters and other related phospholipid metabolites in high concentration, greater than phosphocreatine levels (Table 2). Moreover, the qualitative distribution of the five major phospholipid metabolites is specific of the all species examined.

The concentration of phosphodiesters, measured biochemically, was found to be low in rat heart, and thus not visible in ^{31}P NMR spectra of perfused hearts from this species, whereas it was relatively high (1.8 $\pm$ 0.1 μmol/g-wet weight) in the rabbit heart (8). Glycerophosphorylcholine has been observed in the ^{31}P NMR spectra of the perfused rabbit (8) and ferret (10) hearts, but as one minor peak. Interestingly, high levels of phosphodiesters are present in the ^{31}P NMR spectra of the perfused turtle heart (*Chrysemys picta bellii*) (15). In this paper, we show that high levels of phosphodiesters would be a general feature of the ectotherm heart.

The physiological significance of the presence of these compounds in the ectotherm heart is still unknown. In the mammal heart, glycerophosphorylcholine, glycerophosphorylserine and glycerophosphorylethanolamine represent significant phospholipidic metabolites because the corresponding phospholipids are the major phospholipids of membranes (16). Sarcolemmal phospholipids such as phosphatidylserine being involved in the control of exitation-contraction coupling (16), the elevated turnover of phospholipids could be of critical importance in the physiological adaptation that the heart undergoes in response to a stressor.

The purification and the chemical analysis of the unknown compounds in the fish heart is under study.

Acknowledgments

We thank Nelly Kervarec for her excellent technical assistance and IFREMER (Brest) for the supply of sea breams.

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