NUCLEAR MAGNETIC RESONANCE STUDIES OF AMINE STORAGE IN PIG PLATELETS

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Received 16 November 1981

1. Introduction

Blood platelets contain secretory vesicles that store adenine nucleotides, divalent metal ions, and 5-hydroxytryptamine (5HT) in high concentrations. Nuclear magnetic resonance (NMR) studies of intact platelets have shown that the motional state of vesicular nucleotides and 5HT varies considerably with the species and with temperature. ^{31}P NMR spectra of human platelets, in which the vesicular metal ion is calcium, show extremely broad resonances for vesicular nucleotides between 4–37°C [1–4]. These observations, coupled with the lack of an observable electron diffraction pattern from vesicles [5], have been interpreted as consistent with the presence of an amorphous solid or very high M_r complexes within the vesicles. ^{31}P NMR spectra of pig and dog platelets, in which the vesicular metal ion is known to be predominantly magnesium [6,7], and of bovine platelets show broad resonances from the vesicular nucleotides only at low temperatures; at 37°C, well-resolved resonances can be seen [2–4,8]. These observations suggest that the vesicle contents are in a gel-like state, or that complexes of high M_r exist at 4°C but dissociate as the temperature is raised [4].

The 5HT in platelet vesicles can be studied by incubation of platelets with ring-fluorinated 5HT analogues and examination of the ^{19}NMR spectra [1,3]. These studies suggest that vesicular 5HT in both pig and human platelets experiences a motional state which is very similar to that of the vesicular nucleotides. Here, we present further information on the motional state of vesicular 5HT in pig platelets, and explore by ^{19}F and ^{31}P NMR the relationship between the quantity of 5HT contained in the vesicles and the motional properties of both the 5HT and the adenine nucleotides. In addition, we examine the effects of adding other amines (dopamine and quinacrine) to the vesicles, and compare the results with related studies of human platelets.

2. Materials and methods

Most of the experimental procedures employed here have been documented in [1,3,9]; additional experimental details are recorded below.

3. Results and discussion

When pig platelets were loaded with either 4,6-difluoro-5HT or 6-fluoro-5HT, similar amounts of each analogue were present in the vesicles (as judged from the relative peak heights). The single ^{19}F resonance of 6-fluoro-5HT behaved identically to the corresponding resonance of the ^{19}F in the 6-position of 4,6-difluoro-5HT during temperature changes, being broad at low temperatures and sharper at high temperatures as described for 4,6-difluoro-5HT [1,3]. These data indicated that the electronic environment around the 5-hydroxyl position of 5HT, as well as the pK_a of this group [10], appeared to have little or no influence in either the amount of amine stored or on its motional properties. With both fluorinated 5HTs, the ^{31}P resonances of vesicular adenine nucleotides varied with temperature as described for cells not exposed to fluorinated 5HT [2,3], and thus appeared to be unaffected by the addition of either compound to the vesicles.

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

To estimate a correlation time for intra-vesicular amine, we loaded the cells with 4,6-difluoro-5HT and measured several parameters. The linewidths of the ^{19}F resonances of 4,6-difluoro-5HT varied slightly from one cell preparation to another; at 20°C and 254 MHz, they were 150 ± 25 Hz and 125 ± 25 Hz for the 4- and the 6-positions, respectively. At 94 MHz and 20°C, the corresponding values were 130 ± 20 Hz and 110 ± 20 Hz. Since measured linewidths contain contributions from both magnetic field inhomogeneities and variations in chemical shift position for ^{19}F in different cells, these values may not provide an accurate index of the relaxation behaviour and motional properties of the intra-vesicular 5HT. To examine the contribution of these two factors to the observed linewidths, T_2-values were estimated directly by measuring the decay of the signals in a spin-echo experiment [11]. At 20°C and both 94 MHz and 254 MHz, the T_2-values were 3.1 ± 0.4 ms and 5.4 ± 0.5 ms for the 4- and 6-position, respectively, which correspond to linewidths ($1/\pi T_2$) of 103 ± 15 Hz and 59 ± 6 Hz. The activation energy obtained from T_2-values over 4–30°C was 11.4 ± 3.5 kcal/mol, similar to that estimated in [1,3]. ^{19}F T_1-Values measured using the inversion–recovery method were 0.6 ± 0.1 s at 20°C for both resonances of 4,6-difluoro-5HT, and did not change with temperature. Spin-echo experiments indicated that the ^{31}P resonances of vesicular adenine nucleotides also had short T_2-values, although no attempt was made to quantify the measurements.

The small frequency dependence of the ^{19}F linewidths and T_2-values indicated that the dominant contribution to the transverse relaxation of these nuclei came from dipolar coupling to adjacent nuclei, and that under these conditions field inhomogeneities, chemical shift anisotropy, and chemical exchange contributed $\lesssim$50 Hz to the observed linewidths. If it was assumed that the motion of the 5HT molecule could be described by an isotropic correlation time, its value could be estimated to lie between 10^{-7}–10^{-8} s. This estimate was broadly consistent with the large ratios of T_1/T_2, the short values of T_2, and the absence of large chemical shift anisotropy contributions to T_2. Since the correlation time for free tumbling of a small molecule such as 5HT in aqueous solution is $\sim 10^{-11}$ s, the measurements substantiated interpretations [2–4] that 5HT inside pig platelet vesicles undergoes relatively slow molecular reorientation (i.e., tumbles at a rate much slower than if it were in free solution), even at 20°C.

We also explored the effects of varying the amount of 5HT contained in pig platelets, and of incorporating other amines into the vesicles, on the molecular mobility of the vesicular contents:

(1) The amount of 5HT contained in platelet vesicles were varied by different incubation protocols. Table 1 summarizes data obtained with the same cell preparations used for NMR studies. Incubation of pig platelet-rich plasma (PRP) with 5HT (5×10^{-5} M, 90 min at 37°C) increased the vesicular 5HT content by 50%, and incubation with reserpine (10^{-6} M, 90 min at 37°C) decreased the endogenous content 5HT by 22%. No changes in ^{31}P linewidths or T_2-values of vesicular adenine nucleotides over 4–25°C could be observed following these treatments.

Table 1
Amine content of pig platelets following various incubation procedures

Compound added	Conditions of incubation at 37°C	Net amount of amine present (mol/platelet × 10^{18}, mean ± SEM)	
		5HT	Other amines
None	90 min	14.3 ± 0.1	–
Reserpine, 1 μM	90 min	11.2 ± 0.2 (–22%)	–
X537A, 25 μM	10 min	0.86 ± 0.09 (–94%)	–
5HT, 50 μM	90 min	21.0 ± 0.1 (+50%)	–
[^{14}C]Dopamine, 100 μM	90 min	13.3 ± 0.2 (–7%)	10.2 ± 0.7[a]
Quinacrine, 50 μM	60 min	9.0 ± 0.1 (–37%)	45.7 ± 1.0[a]

[a] As evaluated by the A23187-mediated release of amine [9], essentially all amine was vesicular. A23187 (2 μM) acting for 1 min released 35% of the total vesicles (determined by direct electron-microscopic counts in air-dried whole mounts), 34% of the total 5HT, 32% of the [^{14}C]dopamine, and 34% of the quinacrine

An attempt was made to deplete the pig vesicles of 5HT more completely than with reserpine by treatment with 25 μM X537A (Lasalocid; Aldrich Chemical Co., Milwaukee WI) for 10 min at 37°C. Although the pig platelets lost 94% of their endogenous 5HT content, ^{31}P resonances attributable to vesicular nucleotides were not visible in the NMR spectrum. In [4] vesicles were depleted of 5HT without eliminating the vesicular nucleotides by injection of pigs with reserpine prior to isolation of the platelets; no change in the ^{31}P NMR spectrum was reported [4]. Thus our data are consistent with conclusions that 5HT is not essential for the maintenance of a high M_r complex, aggregate, or gel inside the vesicles of pig platelets [2–4]. Rather, the important components in producing the motional restriction of the vesicular contents in pig platelets appear to be the nucleotides and metal ions. Studies of mixtures of nucleotides and divalent metal ions suggest that magnesium [4,12,13] induces the formation of a gel phase with NMR properties similar to those observed in pig vesicles, while calcium produces a precipitate. Chromaffin vesicles, in contrast, contain much lower levels of either divalent cation than vesicles of pig or human platelets. Both nucleotides and amines in chromaffin vesicles appear from NMR studies to tumble freely in an environment quite similar to that of aqueous solution [14–17].

(2) Amines other than 5HT were incorporated into pig platelet vesicles. Incubation of pig PRP with [^{14}C]dopamine (10^{-4} M, 90 min at 37°C) resulted in the accumulation of a large quantity of this molecule in the vesicles without a dramatic or a corresponding loss of the endogenous 5HT (table 1). The ^{19}F resonance in cells similarly incubated with 5-fluorodopamine was 100 ± 20 Hz at 20°C and 94 MHz, a value very similar to those obtained for the ^{19}F resonances of 4,6-difluoro-5HT.

Pig PRP incubated with quinacrine (5×10^{-5} M, 60 min at 37°C) added considerable quantities of quinacrine to the vesicles with a concomitant loss of 37% of their endogenous 5HT content (table 1). When pig PRP was incubated first with 4,6-difluoro-5HT (5×10^{-5} M, 60 min at 37°C), and then with quinacrine (5×10^{-5} M, 60 min at 37°C) prior to NMR observation, both ^{19}F linewidths had narrowed to 85 ± 20 Hz at 20°C and 254 MHz. The temperature dependence of both linewidths was decreased, reflecting an activation energy of 8 ± 2 kcal/mol. The ^{31}P resonances were also much narrower, with slight shifts in peak position, and could be resolved even at 4°C (fig.1). Human platelets similar loaded with quinacrine showed no ^{31}P resonances attributable to vesicular adenine nucleotides even at 37°C.

These data suggest that both 5HT and dopamine incorporate into vesicles and adopt the motional state of the nucleotide–metal ion complexes [4] without a significant effect on these complexes. Quinacrine, however, is known to complex strongly with adenine nucleotides [18,19], and this interaction may cause partial dissociation of any magnesium–nucleotide

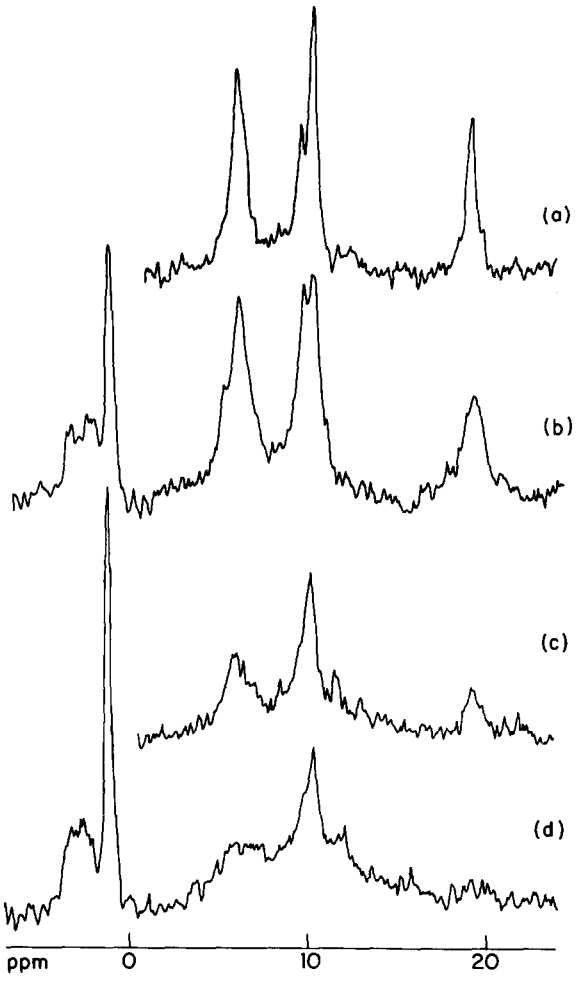

Fig.1. ^{31}P spectra, obtained at 109.3 MHz, of control pig platelets and pig platelets incubated for 60 min at 37°C with 5×10^{-5} M quinacrine. Spectra (a) and (b) were obtained at 25°C, and spectra (c) and (d) at 4°C. Spectra (a) and (c) are of platelets incubated with quinacrine, and spectra (b) and (d) of control platelets.

aggregates responsible for the characteristic motional properties of the pig platelet vesicle interior. In human platelets, where quinacrine has no apparent effect, the quinacrine–nucleotide interaction may not be sufficiently strong to cause appreciable disruption of the more highly aggregated calcium–nucleotide–pyrophosphate structure. The quinacrine-mediated changes in resonances of nucleotides and fluorinated 5HT in pig platelet vesicles demonstrates that the broad linewidths and slow molecular reorientation rates of these species are not merely the consequence of high concentrations of small molecules inside the vesicles. The results also indicate that a restricted motional state of the vesicular contents is not required for efficient storage of either nucleotides or amines.

Acknowledgements

The high-field NMR experiments were performed at the NMR Facility at the Francis Bitter National Magnet Laboratory, MIT. The facility is supported by grant RR0095 from the Division of Research Resources of the National Institutes of Health, and by the National Science Foundation under contract C-670. The work was also supported by the US Navy through Naval Medical Research and Development Command and the Office of Naval Research contract N00014-79-C-0168. The opinions and assertions contained herein are those of the authors and are not to be construed to be official or to reflect the views of the Navy Department or Naval Service at large. F. M. P. acknowledges support from the Danish Natural Science Research Council.

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