

A New Concept: Ionotropin May be a Factor in Mobilization for [a] the Flight or Fight Response and [b] Child Birth

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Abstract

This year we reported the discovery of two classes of steroid phosphoesters: (a) phosphoethanolamine steroid esters (PE-steroids) and (b) phosphocholine steroid esters (PC-steroids). For each steroid, adrenal extracts had both classes of steroids but, in serum, we only detected the PC-steroids. We now describe a possible utility for both classes of steroids. In brief, PE-steroids are synthesized and stored. As part of the response to ACTH, norepinephrine is methylated to epinephrine and PE-steroids are methylated to PC-steroids. Both enzymes require B12. The first step increases glycolysis and the second step increases delivery to muscle by increasing blood pressure. The combined process would be a key factor in the response to stress, such as the 'fight or flight' response and may 'ready' a woman for the stress of child birth. We have already shown that adrenal extracts contain large amounts of PE-steroids. We suggest that the high levels of PC-steroids that we found in cord serum were synthesized from PE-steroids stored in the placenta. The secretion of a specific PC-steroid, possibly Ionotropin, could be an important step in preparing for child birth.

Keywords: *Ionotropin; Spironolactone; N-Methylation; Phosphoethanolamine Steroid N-Methyl Transferase (PESMT); Phosphosteroids; Steroid Phosphoesters*

Abbreviations

PE-Steroids: Phospho-Ethanolamine Steroid Esters; PC-Steroids: Phospho-Choline Steroid Esters; PS-Steroids: Phospho-Serine Steroid Esters; PMT-1: Phosphoethanolamine Methyl Transferase; PEMT-1: Phosphatidyl-Ethanolamine N-Methyl Transferase; PESMT: PE-Steroids N-Methyl Transferase; ACTH: Adrenocorticotrophic Hormone; CK: Choline Kinase; EK: Ethanolamine Kinase; SLOS: Smith-Lemli-Opitz Syndrome

Introduction

This is the third paper in the series describing the discovery of a new class of steroids and their function. The first paper detailed the initial discovery of Ionotropin and concentrated on the biosynthetic pathway of the steroid moiety [1]. The second paper described the role of Ionotropin in the post-natal period, showing the role played by Ionotropin as the infant accommodates to independent living [2]. This paper, the third paper in the series, describes (a) the origin of the phosphoester component and (b) proposes an explanation for the high concentrations of the PE-steroids at the site of synthesis.

Ionotropin

This year, we reported the isolation of Ionotropin from mammalian sources. Ionotropin can be detected as a digoxin-like material (DLM) [1]. It is the phosphocholine ester of a steroid with 23 carbons. There are no previously known mammalian steroids with 23 carbons nor any other known PC-steroids. Ionotropin shares structural features with spironolactone and may be its endogenous equivalent. There were three other PC-steroids in serum. In addition to the PC-steroids, in bovine adrenal extracts we also identified a PE-steroid for each of the same steroids. We proposed that the 8 compounds are related and form a biosynthetic pathway to Ionotropin.

Phosphatidyl choline and phosphatidyl ethanolamine biosynthesis

Although there are many known diacyl glycerol phosphodiester, there are no known sterol phosphodiester. The main pathway to phosphatidyl lipids is often described as the *de novo* pathway, but *de novo* does not refer to biosynthesis of choline or ethanolamine [3].

Neither choline nor ethanolamine are synthesized *de novo* in mammalian cells but must be obtained from dietary sources. When the amines enter mammalian cells, choline kinase (CK) (EC 2.7.1.32) and/or ethanolamine kinase (EK)(EC 2.7.1.82) are the first steps in the pathway. CK phosphorylates both amines whereas EK reacts primarily with ethanolamine [3]. The next step is the activation of the amine phosphate by condensation with CTP to form CDP-choline or CDP-ethanolamine [3]. The CDP esters have a high energy bond which activates the phosphoamine for transfer to an acceptor. Kennedy recognized that CDP-choline condenses with to sn-1,2-diacylglycerol to form phosphatidyl-choline (See figure 1), the ‘Kennedy’ pathway. Kennedy also described a similar step for the condensation of CDP-ethanolamine to form phosphatidyl-ethanolamine [4]. Finally, phosphatidyl-ethanolamine can be methylated to form phosphatidylcholine [5]. The methyl group is contributed by S-adenosyl-methionine in a B12 dependent process. A 3rd pathway proceeds from condensation of CDP-serine to form phosphatidyl serine and decarboxylation to phosphatidylethanolamine. The 2nd and 3rd pathways differ in some unknown way because the lipid distribution of the phosphatidyl ethanolamine differs, depending on the pathway [5]. This 3rd pathway may be the dominant mammalian pathway as serine is not a required nutrient but is available as a common metabolic intermediate. Figure 2 shows the structure of the three hydroxyamines used as polar ‘head’ groups in phosphatidyl-lipids.

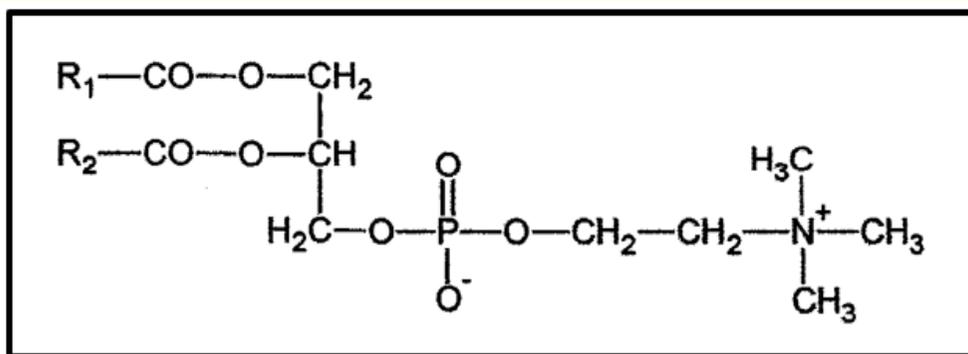
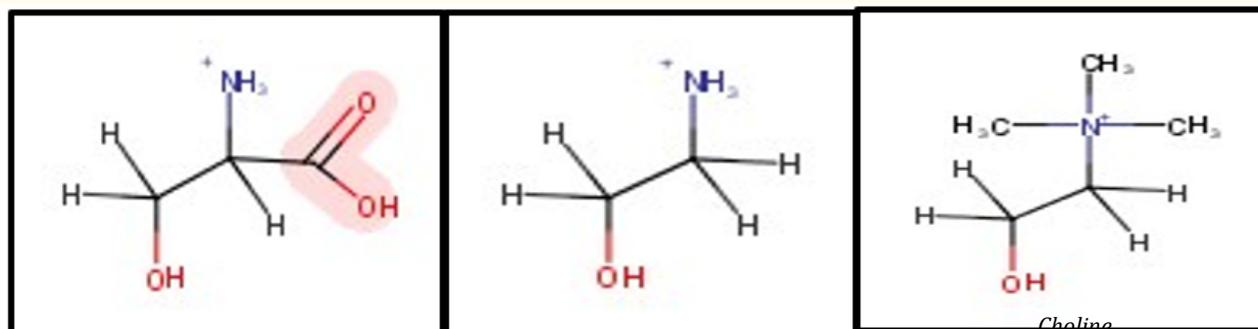


Figure 1: Phosphatidyl choline.



Serine

Ethanolamine

Choline

Figure 2: The ‘head’ groups of phosphatidyl lipids and sterol phosphoesters.

Biosynthesis of PC-steroids and PE-steroids

At the present, there are no data describing the pathway for the synthesis of the phosphocholine component of Ionotropin. We have identified PE-steroids for each of the PC-steroids. Our hypothesis is that the synthesis of PC-steroids is similar to the synthesis of phosphatidyl choline as described in section 4.2. If PC-steroids and PE-steroids are not part of the same pathway, we have more to discover.

PMT-1 vs PESMT

PMT-1 is the enzyme that synthesizes phosphatidyl choline by N-methylation of phosphatidyl ethanolamine. PMT-1 requires B12 and uses S-adenosyl methionine as a methyl donor. Although the process requires three methylations, the intermediate products are preferentially methylated, leading to phosphatidyl choline. The conversion of norepinephrine to epinephrine has similar requirements but the reaction only proceeds to the N-methyl stage. This points to multiple enzymes catalyzing the ACTH dependent- methylation because the substrates and products are significantly different.

PESMT would be the corresponding enzyme that N-methylates the PE steroids, including the precursor of Iontropin. This is consistent with the absence of N-methyl or N,N-dimethyl phosphoethanolamine steroid esters in adrenal extracts. However, nothing is known about this enzyme because the PE-steroids were first described this year and the two functions (PMT-1 and PESMT) need not be catalyzed by the same protein.

Significance of B12 and B12 deficiency

There are about a dozen enzymes that require B12 (cobalamin) as cofactors. The enzymes can be divided into two general groups: mutases and methyl transferases. When women are pregnant, they require large amounts of B12 and folate. B12 deficiency is most common in vegans because plants do not make B12. B12 and/or folate deficiency in pregnancy leads to a 5-fold increase in neural tube defects such as spina bifida [6]. The pathology that leads from B12 or folate deficiency to spina bifida has not been elucidated.

Methods and Results

Extraction, sample preparation, LC-MS analysis

Samples for analysis were mixed with acetonitrile and filtered with Whatman Syringe nylon filters with 0.2 μm pore size. The column used was an amino carbohydrate column purchased from All-tech, Chicago, IL (3.9 mm x 300 mm, 10 μm). The MS was operated in APCI positive ion mode; cone voltage 75 volts; 180 C. Nitrogen was the nebulizing gas. Under these LC-MS conditions, PC-steroids do not fragment to generate a steroid fragment. The LC-MS work station used MassLynx software. Table 1 shows the gradient used for analytical purposes. At the completion of the gradient, the column had returned to its starting solvent and a new sample was injected with the auto-injector. Data is presented as generated by the software [1].

Time	A (%)	B (%)
0	5	95
2	25	75
12	45	55
14	60	40
16	60	40
17	5	95
20	5	95

Table 1: Description of LC gradient.

Solvent composition: A= Water/Acetonitrile (99:1); B= Acetonitrile; Flow = 0.5 ml/min;

Injection loop volume = 10 μl .

Analysis of phosphoesters in human serum

Figure 3 shows LC-MS analysis of PC-steroids from a patient with hGH deficiency under therapy. The details of the sample collection are included in Chasalow and Pierce-Cohen [1].

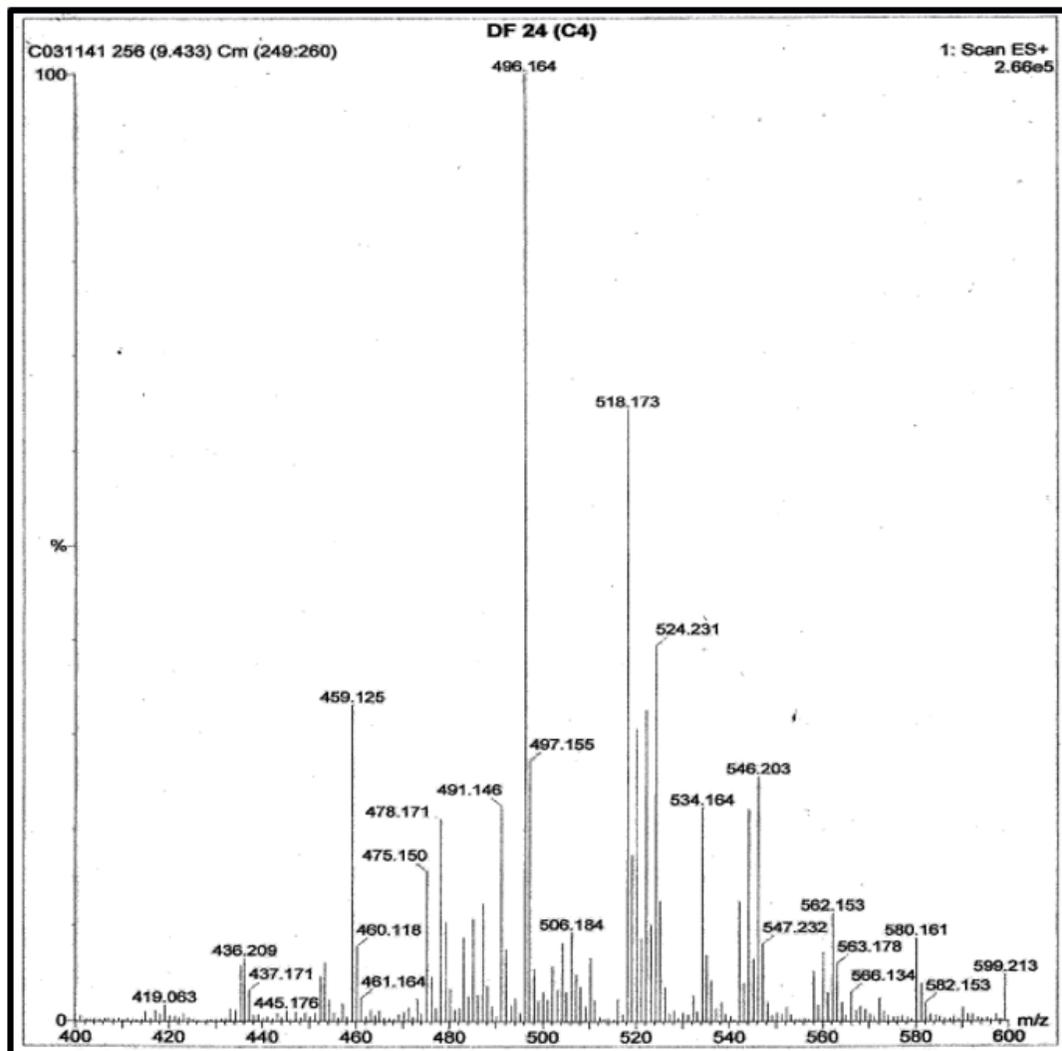


Figure 3: LC-MS from a 12-year old boy with hGH deficiency under therapy.

Details are in section 5.2.

- The ^{12}C isomer has 99% abundance but there is also a ^{13}C isomer with an abundance of 1.1%. This causes a shadow peak at +1 Da for every mass ion. The shadow peak for Iontropin has a height about 30% of the main peak because there are 30 carbon atoms in Iontropin - 23 carbons in the steroid and 7 carbons in the choline, any one of which can be a ^{13}C isomer.
- The main chromatographic peak for the PC-steroids was at a retention time of 9.4 min. The chromatography conditions were not intended to resolve the 4 PC-steroids and did not do so. Identity was evaluated by the MS results.

- The biggest peak identified by LC-MS is the PC-steroid attributed to the M+1 ion of Δ^5, Δ^7 -17OH-pregnanolone at 496 Da. The 518 Da peak is the sodium salt and the 534 Da peak is the potassium salt. The peak at 459 Da is attributed to loss of the trimethylamine fragment from the sodium ion. The ^{13}C peaks are at 497 Da, 519 Da and 535 Da. If there was a PE-steroid for this compound, the M+1 ion would be at 454 Da. The corresponding Na⁺ and K⁺ ion forms would be at 476 Da and 492 Da, respectively. No significant ions were present at any of these values.
- For Ionotropin, the LC-MS identified 3 peaks: M+1 – H⁺ ion form; M+23 indicating the Na⁺ ion form; and M+39 indicating the K⁺ ion form. The molecular weight of Ionotropin is 523 Da. Thus, the Ionotropin peaks are at 524 Da, 546 Da, and 562 Da. The ^{13}C peaks are at 525 Da, 547 Da and 563 Da. The mass ions for the PE-steroid would be at 482 Da, 504 Da, and 520 Da.
- For the two precursors of Ionotropin, careful examination of mass spectrum confirms the three-peak pattern for the PC-steroid and the absence of the similar one that would be attributed to the corresponding PE-steroids, if they were present.
- The data shown in figure 3 is representative of 39 pediatric samples collected in the normal course of patient care. Diagnoses included diabetes, hypothyroidism, hyperthyroidism, short stature and growth hormone deficiency. Pediatric patients were selected because of the general guideline of “one disease to a customer”.

Overall, LC-MS analysis of human serum demonstrated the presence of four PC-steroids but PE-steroids were not present. The same was true for serum from other mammals.

Analysis of phosphoesters in extracts from bovine adrenals

Chasalow and Pierce-Cohen isolated the phosphoesters from bovine adrenal extracts. Bovine adrenals were homogenized and extracted with 3 volumes of acetonitrile. The extract was filtered and applied to a preparative HPLC column (20 mm x 500 mm). Application of sample was stopped when the silver nitrate spot test on the column eluate was positive. The phosphoesters were then eluted with a gradient similar to the analytical gradient. Each fraction was analyzed by LC-MS. The PC-steroids eluted in fraction 19. The PE steroids eluted in fraction 22. Figure 4 shows the LC-MS chromatogram obtained at a retention time of 9.8 minutes.

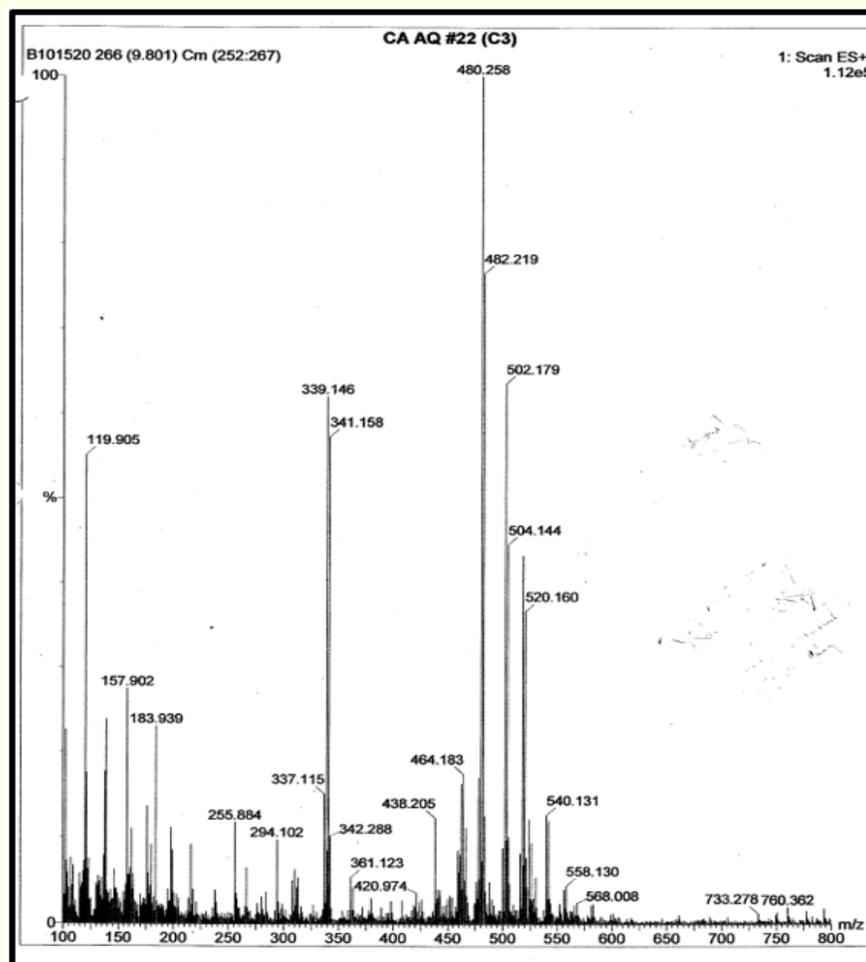


Figure 4: Analysis of phosphoesters in extracts from bovine adrenals.

Details are in section 5.3.

- The limited peak height of the fragment at 184 Da confirms the limited amount of PC-steroids present in fraction 22. This is also confirmed by the absence of peaks at 524 Da, 546 Da, or 562 Da which are characteristic of Ionotropin.
- The LC-MS peaks at 482 Da, 504 Da and 520 Da would be predicted to indicate the PE-steroid of Ionotropin. The 341 Da fragment is also observed with Ionotropin at high voltages. Apparently, the PC steroid is less susceptible to fragmentation than is the PE steroid.
- The LC-MS peaks at 480 Da, 502 Da and 518 Da would be predicted to derive from the PE-steroid of $\Delta 5$ -Ionotropin. The 339 Da fragment is also observed, just like the corresponding PC-steroid.
- Thus, the MS obtained at 9.8 minutes shows a mixture of two of the PE-steroids. The MS obtained at a retention time of 10 minutes (not shown) indicates a mixture of all three PC-steroids with 23 carbon atoms.
- Overall, this experiment shows the presence of large amounts of PE-steroids in adrenal extracts.

So far, we have isolated phosphoesters of 6 different steroids. We have identified the structure of 4 of these steroids (See table 2). In bovine adrenal extracts, for each steroid, there was both a PC steroid and a PE steroid. However, only the PC-steroids were detectable in individual human serum samples.

Steroid		MW*(Da)	PC		PE	
Ionotropin	(C ₂₃ H ₃₄ O ₃)	358	524	X	482	ND
$\Delta 5$ - Ionotropin	(C ₂₃ H ₃₂ O ₃)	356	522	X	480	ND
$\Delta 5, \Delta 7$ - Ionotropin	(C ₂₃ H ₃₀ O ₃)	354	520	X	478	ND
Pregna- $\Delta 5, \Delta 7$ -dien-17 α -olone	(C ₂₁ H ₃₀ O ₃)	330	496	X	454	ND
$\Delta 5$ Unknown A #	(C ₂₃ H ₃₈ O ₄)	378	544	X	502	ND
Unknown B #	(C ₂₃ H ₄₀ O ₄)	380	546	X	504	ND

Table 2A: PC-Steroids and PE-Steroids in human serum.

Steroid		MW (Da)	PC		PE	
Ionotropin	(C ₂₃ H ₃₄ O ₃)	358	524	X	482	X
$\Delta 5$ - Ionotropin	(C ₂₃ H ₃₂ O ₃)	356	522	X	480	X
$\Delta 5, \Delta 7$ - Ionotropin	(C ₂₃ H ₃₀ O ₃)	354	520	X	478	X
Pregna- $\Delta 5, \Delta 7$ -dien-17 α -olone	(C ₂₁ H ₃₀ O ₃)	330	496	X	454	X
$\Delta 5$ Unknown A	(C ₂₃ H ₃₈ O ₄)	378	544	X	502	X
Unknown B	(C ₂₃ H ₄₀ O ₄)	380	546	X	504	X

Table 2B: PC-Steroids and PE Steroids in bovine adrenal extracts.

Notes to table 2A and 2B

* = The molecular weight (MW) of each compound was determined by adding 17 to the steroid fragment obtained on LC-MS. Each molecular weight could be attributed to a unique composition.

Δ = Indicates the presumed location of alkenes in the steroid portion of phosphoesters.

#= Detected in turkey serum

X= Detected by LC-MS ND= Not detected by LC-MS

Unknown A and B = These are both steroids with 23 carbon and 4 oxygen atoms but the specific structure has not yet been determined.

Unknown A has one double bond, possibly as a $\Delta 5$ -6 alkene and Unknown B seems to be an alkane.

Discussion

Why are there both PC-steroids and PE-steroids in adrenal extracts but only PC-steroids in serum?

This paper can be divided into two parts. Part 1 documents [a] PC-steroids in serum and [b] both PC-steroids and PE-steroids in adrenals. Part 2 proposes a new concept. In response to stress, PESMT methylates PE-steroids to the corresponding PC-steroid, which are secreted into the serum. This process is a key part of the stress response as it provides an increased glucose supply to muscles and brain.

Isolation of steroid phosphoesters

Initially, human breast cyst fluid was extracted and purified by HPLC [6]. The purification was monitored by DLM assay [1] and a silver nitrate spot test. LC-MS analysis showed that the active fractions had peaks at $m/z = 524, 546, \text{ and } 562$ Da. The pattern suggested the three peaks were the result of three different cations: H^+ , Na^+ , and K^+ . There was also a fragment at $m/z = 184$ Da. Model compounds showed that this pattern was characteristic of phosphocholine lipids.

Human breast cyst fluids were not a suitable source for isolation of unknown compounds. We obtained outdated human blood and repeated the isolation. As illustrated in figure 3, there were four different compounds that had spectra characteristic of phosphocholine esters. The 1H -NMR spectra were similar to that observed with authentic steroids and ^{31}P -NMR confirmed the presence of a phosphate ester. The $m/z = 184$ Da confirmed phosphocholine as the phosphate ester.

In the next phase, I obtained bovine adrenals and repeated the isolation on a preparative scale. In addition to the PC-steroids at a retention time 9.2 minutes, there was a second group of phosphoesters at a retention time of 9.8 min. The pattern showed three cations were present, indicating H^+ , Na^+ and K^+ , but did not have the $m/z=184$ Da fragment. The m/z ions of the acceptor were all present – $m/z = 341, 339, 337$ and 313 Da. The pattern indicated that the m/z ions were all 42 Da less than the PC-steroids. This is consistent with the pattern expected for PE-steroids and was confirmed by model compounds.

Molecular composition of the PC-steroids and PE-steroids

LC-MS fragment analysis enabled evaluation of the chemical formula of each of the phosphoesters. Phosphoesters were recognized by the triad of three ions: $M, M+22, \text{ and } M+38$, attributed to $H^+, Na^+ \text{ and } K^+$ ions, respectively. Phosphoesters that contained an $m/z = 184$ fragment were recognized as PC-steroids. This fragment was also observed in model compounds containing a phosphocholine 'head' group. In addition to the $m/z = 184$ Da fragment, all of the PC-steroids had a fragment in 300 - 400 Da range (See figure 4). This fragment must contain the remaining portion of the molecule. When combined with the $m/z = 184$ fragment, the resulting value coincided with the m/z of the phosphoester. Thus, the 300 - 400 Da fragment was the 'acceptor' steroid.

In the second stage of the analysis, 17 Da was added to the observed m/z value for the acceptor fragment. The completed acceptor molecule had an "even" formula mass, as would be expected for a molecule containing carbon, hydrogen and oxygen atoms, but not nitrogen or phosphorous. Trial and error analysis identified all compositions that would lead to a satisfactory molecule. For each compound, only one composition satisfied the valence conditions. The proposed chemical formulas are shown in table 2. The observation of only one composition was a surprise. It doesn't imply that there is only one possible isomer. However, it does determine the number of rings and double bonds.

Substrate requirements for synthesis of phosphoester steroids

Of the 6 PC-steroids we isolated from adrenal extracts, two were Δ^5 - Δ^7 dienes, two were Δ^5 steroids and two were alkanes. Five of the compounds had 23 carbon atoms, but no mammalian steroids with 23 carbons have been reported. Second, there are enzymes that reduce steroidal alkenes but there are no enzymes that introduce double bonds into steroids. Thus, the synthetic pathway must go from the Δ^5 - Δ^7 dialkene to the Δ^5 alkene and then to the alkane (Occam's razor).

The recognition that the acceptor for the CDP-ethylamine donor is a dialkene steroid implicates a second enzyme with a different substrate specificity than the enzyme that makes phosphatidyl lipids because the $\Delta 5$ - $\Delta 7$ dialkene steroid structure forms a rigid plane rather than the floppy structure of the *sn*-1,2-diacylglycerol used for the synthesis of phosphatidyl lipids. This will have to be confirmed by a specific experiment.

Which CDP donor is used for the synthesis of Phosphosteroid esters

Figure 2 shows the three amine precursors that can be linked to CDP and used for the synthesis of phosphatidyl lipids. Although choline and ethanolamine are generally considered to be essential nutrients, both choline and ethanolamine can be obtained by hydrolysis from phospholipids. We have no direct evidence - pro or con - of CDP-serine as a substrate for condensation with steroids. However, CDP-serine would seem to be the prime candidate because serine-phosphate is not an essential nutrient and can be synthesized as needed. In the proposed pathway, CDP serine donates serine phosphate to a sterol to form PS-steroid. This would be analogous to the synthesis of phosphatidyl-serine. The serine containing compounds would be decarboxylated to the phosphoethanolamine in a B6- dependent process. The final synthesis of the phosphocholine steroid would be catalyzed by three sequential methylases catalyzed by a B12-dependent S-adenosyl-methionine. PS-steroids were not detected in either serum or adrenal extracts. However, a PS-steroid would be expected to be more anionic than a PC-steroid and might not be detected with methods developed for PC-steroids or PE-steroids. This will also have to be confirmed by a specific experiment.

Serum PC-steroids

Although both PC-steroids and PE-steroids are present in adrenal extracts, only PC-steroids are present in serum. This implies that the PE-steroids may not be secreted but are only a storage form. This points to regulation of N-methylation as a key step in Ionotropin synthesis and function.

Summary of biosynthesis

There are still some gaps in the pathway leading to Ionotropin. The donor of the polar head group could be CDP-serine, CDP-ethanolamine, or CDP-choline. The acceptor seems to be a $\Delta 5$ - $\Delta 7$ dialkene. For Ionotropin, the precursor is pregna- $\Delta 5$, $\Delta 7$ -diene-17 α -olone. Shackleton showed this compound was present in patients with $\Delta 7$ -sterol reductase deficiency (SLOS) [8].

Synthesis of Ionotropin is a multiple step process with potential regulation at more than one step. The starting substrate is a $\Delta 5$ - $\Delta 7$ diene steroid. 7-dehydrocholesterol can be reduced to cholesterol but is also a substrate for side-chain cleavage and 17 α -hydroxylase, leading to pregna- $\Delta 5$, $\Delta 7$ -dien-17 α -olone [9]. Based on the compounds we isolated in bovine adrenal extracts, pregnadienolone is the first steroid in the pathway that serves as an acceptor for CDP-ethanolamine. We did not detect any PS-steroids but they might also be a precursor. PE pregnadienolone could be further metabolized to other steroids and to PC-pregnadienolone, all of which were detected in adrenal extracts. However, no PE-steroids were detected in serum. The structure, biosynthesis and function of Unknown A and B have not yet been identified.

Relationship to spironolactone

Spironolactone is a synthetic potassium sparing diuretic. Ionotropin shares structural features with spironolactone and would be expected to share functions. Spironolactone has two functions: (a) recovers potassium in the kidney and (b) increases blood pressure. If Ionotropin has the same functions, it could be a key element in the stress response. ACTH could stimulate N-methylation of PE-steroids to PC-steroids, including Ionotropin. In turn, increased Ionotropin would lead to increased glycolysis in the liver and, by increasing blood pressure, increase availability of glucose to muscles. Both steps could be part of the physiology.

B12 deficiency is known to contribute to congestive heart failure. Congestive heart failure can be treated with spironolactone. Inadequate conversion of PE-steroids to PC-steroids might be part of the pathology.

Biosynthesis of other candidates for endogenous DLM - Endogenous Ouabain

There are several other candidates for the endogenous DLM[10]. The one that has been most investigated is ouabain [11]. Biosynthesis in adrenal extracts of ouabain has been reviewed [12]. Investigators detected increased cross reacting material (DLM) with an immunoassay specific for ouabain but did not characterize their products by any physico-chemical process. For example, (1) the newly synthesized endogenous ouabain was not extracted, chromatographed or shown to co-elute with authentic ouabain or (2) radio-labeled precursors (perhaps cholesterol or pregnenolone) were not tested to determine if they were incorporated into the newly synthesized 'ouabain.' Nicholls suggests that, without direct physicochemical evidence of precursors, the claim for ouabain as an endogenous hormone remains unconfirmed [13].

In contrast, although 3 of the PC-steroids that we isolated can be detected by DLM immunoassays, they are clearly not digoxin nor ouabain. The corresponding PE-steroids may also be DLM, depending on the specificity of the specific antibody. In summary, our isolation of Ionotropin and the other steroid phosphoesters satisfies Nicholls' requirement of precursors for an endogenous cardiotonic steroid candidate.

Conclusion

The distribution of PC-steroids and PE-steroids leads to a new concept in ACTH function. The occurrence of the PE-steroids, including Ionotropin, in adrenal extracts points to PE-steroids as a precursor form that was stored in the tissue, awaiting methylation by PSEMT. The occurrence of the PC-steroids in the adrenal could be the result of the method of collection of the adrenals. For example, the cattle might release ACTH in response to the smell and processing in the slaughterhouse. In turn, ACTH would stimulate N-methylation of nor-epinephrine to epinephrine and of PE-steroids to PC-steroids, including Ionotropin. Mobilization of glucose from glycogen in the liver in response to epinephrine and increase in blood flow (pressure) in response to Ionotropin, together, would be desirable as part of a fight or flight response.

Speculation - new concept about the role of Ionotropin during child birth

Of all of the serum samples examined, the serum from the placental 'cord' has by far the highest concentration of PC-steroids, more than ten times the amount detected in normal human serum (2). We predict that the high levels of PC-steroids we found in cord serum were synthesized from PE-steroids in the placenta. The secretion of a specific PC-steroid, possibly Ionotropin, could be an important step in preparing for child birth.

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Conflict of Interest

No funding from any source.

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