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COMPLEXATION OF OKADAIC ACID : A PRELIMINARY STUDY

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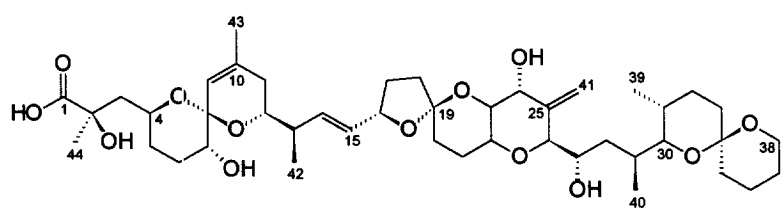
Abstract: A complexation study was carried out with okadaic acid (OA) and the univalent metal ions Li^+, Na^+ and K^+, and the divalent metal ions $Ca^{2+}$and Mg^{2+}. K^+ binding was observed identical with a complex obtained from the natural source (OAC). The pharmacological trials demonstrated that this cation has a very important influence on the pharmacological activity of okadaic acid.

Introduction

Okadaic acid (OA) **1**, (Figure 1), is responsible, together with other polyether derivatives, for the diarrhetic syndrome known as DSP[1] that, though not fatal, causes a great impact on public health and in the food industry when the toxic periods are extended. It has been found to be a potent and highly selective inhibitor of protein serine/threonine phosphatases type 1 (PP1) and type 2A (PP2A)[2] providing a useful tool for investigating the role of protein phosphorylation in different cellular signal transduction mechanisms. OA has also been reported to produce smooth muscle different and interesting biphasic response.[3]



Okadaic acid (OA) **1**

Figure 1

When we isolated OA in our laboratory, **Figure 1,** from the unialgal culture of the marine dinoflagellate *Prorocentrum lima*, it generally appears complexed to a metallic cation.[4] The samples of this complex could not be differentiated from the free samples during the chromatographic process, because their

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chromatographic behaviours under the conditions used were identical. However, the two samples showed significantly different ^{1}H-NMR spectra: the metallic complex showed a much poorer resolution, undergoing individual chemical shift differences (**Tables 1 and 2**). Treatment of this sample with a few drops of EDTA disodium salt solution (80 mg/ml), yielded pure free okadaic acid (**Figure 2**).[5] We therefore decided to carry out a series of experiments in order to establish the nature of this metallic cation.

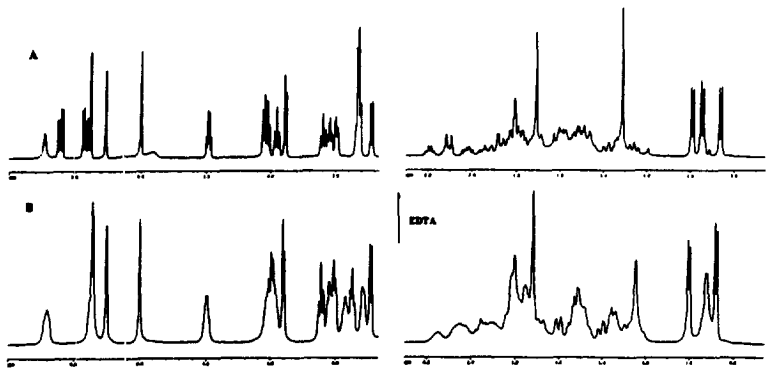

Figure 2. Comparison of the ^{1}H-NMR (600 MHz) spectra of okadaic acid , OA (A) and the complexed OA (B) in $CDCl_3$.

Material and Methods

1.- Complexation studies

Experiment A:

To 0.5 ml of a solution 5.58 mM (2.79 mmol) of OA in $CDCl_3$ was added 0.4 ml of the D_2O saline solution 200 mM (80 mmol) of the corresponding cation chlorides (LiCl, NaCl, KCl, $CaCl_2$ and $MgCl_2$), dried in vacuo in the presence of P_2O_5 before use. The resulting mixture was submitted to vigorous stirring during 5 min and centrifugation. Once the organic phase was separated, it was monitored by ^{1}H NMR spectroscopy and compared with a free okadaic acid solution prepared as standard. This experiment was repeated, increasing the saline concentration by using 400 mM and 600 mM solutions.

Experiment B:

OA (2.79 mmol) and the corresponding chlorides (20 mmol) were dissolved in 5 ml of the mixture MeOH:H_2O (85:15). They were then passed through a Sep-pak C-18, washed subsequently with 5 ml of MeOH, the solvents evaporated, redissolved in $CDCl_3$ and monitored by ^{1}H-NMR spectroscopy.

2.- Treatment of OAC with crown-ethers

To two samples of 1.5 mg each of OA complex obtained from natural source (OAC) and dissolved in 0.4 ml of $CDCl_3$, were added 50 µl of solutions in $CDCl_3$ (0.1 M) of the following crown ethers:18-Crown-6 (1,4,7,10,13,16 -hexaoxacyclooctadecane) and 15-Crown-5 (1,4,7,10,13 -pentaoxacyclopentadecane). The solutions were submitted to stirring and monitored by ^{1}H NMR spectroscopy.

Table 1. ^{1}H-NMR (400 MHz) δ: (a) Free Okadaic Acid (OA). (b) Complex Okadaic Acid (OAC).

H	a	b	H	a	b	H	a	b
3	2.12	2.08	18	1.80	1.85	33	1.52	1.55
3'	1.62	1.56	18'	2.04	2.05	33'	1.34	1.37
4	3.96	3.98	20	1.32	1.36	35	1.31	1.36
5	1.72	1.72	20'	1.47	1.49	35'	1.48	1.49
5'	1.31	1.27	21	1.81	1.83	36	1.39	1.41
6	1.79	1.78	21'	1.72	1.76	36'	1.61	1.61
6'	1.83	1.82	22	3.57	3.58	37	1.84	1.84
7	3.34	3.32	23	3.35	3.40	37'	1.51	1.53
9	5.29	5.28	24	4.07	4.07	38	3.62	3.65
11	1.87	1.80	26	3.90	3.91	38'	3.53	3.53
11'	1.91	1.95	27	4.04	4.01	39	0.88	0.89
12	3.35	3.47	28	0.95	0.93	40	1.01	1.03
13	2.21	2.17	28'	1.28	1.34	41	5.39	5.38
14	5.63	5.74	29	1.91	1.91	41'	5.02	5.03
15	5.42	5.38	30	3.25	3.28	42	0.97	0.93
16	4.51	4.52	31	1.75	1.79	43	1.73	1.74
17	2.14	2.06	32	1.96	1.98	44	1.36	1.27
17'	1.54	1.54	32'	1.86	1.83			

Table 2. ^{13}C-NMR (100 MHz) δ : (a) Free Okadaic Acid (OA). (b) Complex Okadaic Acid (OAC).

n°C	a	b	n°C	a	b	n°C	a	b
1	177.5	176.7	16	79.60	80.2	31	27.83	28.1
2	77.02	77.2	17	31.05	31.3	32	26.77	27.0
3	42.84	44.5	18	37.73	38.0	33	30.78	31.0
4	69.75	69.9	19	106.09	106.5	34	96.02	96.3
5	31.98	32.5	20	33.22	34.0	35	36.32	36.6
6	27.53	27.0	21	27.02	28.1	36	19.18	19.4
7	71.96	72.5	22	70.16	70.6	37	25.86	26.1
8	96.96	96.9	23	76.86	76.6	38	60.77	61.0
9	121.85	122.8	24	71.55	71.8	39	11.14	11.3
10	139.85	139.4	25	145.22	145.7	40	16.61	16.9
11	33.64	33.7	26	85.29	85.3	41	112.97	113.2
12	71.96	71.6	27	64.98	65.7	42	16.31	16.7
13	42.63	43.3	28	36.65	36.0	43	23.54	23.9
14	136.85	138.7	29	31.50	31.7	44	27.69	29.0
15	131.76	131.0	30	75.46	75.8			

3.- Animals and tissue preparation

Myometrial tissue was obtained from virgin female Wistar rats (200-250 g) pretreated with 17β-estradiol benzoate (100 μg Kg^{-1} ip) 24 h. before the experiments. The estrus stage was confirmed by microscopic examination of vaginal smears. Longitudinal strips of uterine smooth muscle (8-10 mm in length and 2-3 mm in width) were prepared and mounted on isolated tissue baths containing 4 ml of Sund's physiological salt solution (PSS). The preparations were bubbled continuously with 95% O_2, 5% CO_2 and warmed to 32°C.

Changes in isometric tension were recorded by means of force-displacement transducers (Grass FT-03) connected to a LETICA amplifier and an ABB GOERZ SE 130 multichannel recorder.

4.- Physiological solutions

Sund's physiological salt solution (PSS) contained (mM): NaCl 154; KCl 5.6; $CaCl_2$ 0.54; $MgCl_2$ 0.95; $NaHCO_3$ 5.95 and glucose 2.78 (pH 7.4). The Ca^{2+}-free solution (Ca^{2+}-free PSS) was prepared by omitting $CaCl_2$ and adding ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, 3mM). The Ca^{2+}-free, Na^+-free solution was prepared by replacing NaCl with equimolar KCl in Ca^{2+}-free PSS. The Ca^{2+}-free, K^+-free solution was obtained by omitting KCl from the Ca^{2+}-free PSS without any substitution.

5.- Protocols

The preparations, stretched to the optimal resting force of 0.5 g, were equilibrated for 45 min and then repeatedly stimulated with a maximally effective concentration of acetylcholine (ACh, 1 mM) until reproducible responses was obtained. The last response served as an internal standard for all experiments. Uterine strips were then allowed to equilibrate for a further 60 min period in one of the following solutions: a) Ca^{2+}-free PSS; b) Na^+-free, Ca^{2+}-free PSS or c) K^+-free, Ca^{2+}-free PSS and then OA or its complex derivative was added. Only one concentration of OA or its derivative was applied to each strip since we found in previous experiments that OA cannot be removed by washing. The concentration of OA (20 μM) was chosen from previous experiments which revealed that this concentration induced a contractile response of similar amplitude to those evoked by maximally effective concentrations of ACh (1mM) and oxytocin (1 μM). OAC was also used at this concentration for comparison. The maximal contractile effect (E_{max}) was expressed as a percentage of the maximal tension evoked by ACh 1 mM. Other parameters were obtained for characterizing the biphasic response to OA and OAC: time to peak tension, duration of the plateau of contraction and % of relaxation at a given time, and were calculated by considering as 100% the maximal increase in tone induced by the polyether.

6.- Expression and statistical analysis of results

All values in the text and tables are expressed as mean ± s.e. mean for *n* number of experiments. Statistical significance of differences between two means was assessed by Student's test for unpaired data. Multiple means were compared by one-way analysis of variance (ANOVA). P values of less than 0.05 were considered to represent significant differences.

Results and Discussion

The configurational and conformational features of the free okadaic (OA) acid and its complex (OAC) have been studied from scalar and dipolar coupling connectivities extracted from 2D-NMR experiments, obtained from the two samples.[4,6] The hypothesis of the presence of a cation in the zone of the molecule C1/C26 agrees with the two experimental facts: the diol esters isolated from the *Prorocentrum lima* culture,

have never been isolated from a natural source as a complex;[7] if the chemical shifts of the proton NMR spectra of OA and OAC are compared, it can be observed that the most notable differences correspond to the signals of the double bond H-14/H-15, the methines H-12; H-23 and the secondary methyl group Me-41.

On the base to these considerations, we decided to test samples of OA with different metallic cations, in order to establish its nature in OAC. When choosing these metallic cations for the trials, the size of the cavity adopted by OA in solution[6] was considered as well as the ions recognized as influencing on the cellular processes, Li^+, Na^+, K^+, Ca^{2+} and Mg^{2+} being those which combine both requirements.

These experiments were carried out according to the methodology described in Material and Methods (Experiments A and B). Attempts to obtain OAC through experiment A failed, as deduced by comparison of the ^{1}H-NMR spectra of OA and those of the samples obtained, which showed the absence of significant changes in all cases. However, we observed frequently in our chromatographics studies, that non-complexed toxin samples submitted to purification in reverse-phase column chromatography with MeOH:H_2O (85:15) returned to a complex form. We therefore deduced that, perhaps, for the total complexation of the sample, a physical support was necessary. Thus, in Experiment B, samples of OA (2.23 mmol) and cation (20 mmol) were dissolved in 5 ml of MeOH:H_2O (85:15) and passed through a Sep-pak C-18, washing with 5 ml MeOH. The ^{1}H-NMR analysis showed light variations for the samples treated with salts of Li^+ and Na^+, while no change was observed after treatment with salts of Ca^{2+} and Mg^{2+}. However, in the case of the sample treated with potassium salt, its ^{1}H-NMR turned out to be identical with that of OAC. This process was repeated with a larger amount of sample in order to accomplish a ^{13}C-NMR spectrum that was also totally equivalent to that of OAC.

The presence of potassium in OAC was reinforced by the treatment of samples of OAC with the crown ethers 18-Crown-6 (1,4,7,10,13,16 -hexaoxacyclooctadecane) and 15-Crown-5 (1,4,7,10,13 -pentaoxacyclo pentadecane), which showed that the sample treated with the ether 18-Crown-6 turned out to be totally and immediately non complexed, while it was mainly unaffected by the ether 15-Crown-5.

On the other hand, OA and OAC (20 μM) caused similar contractions in both amplitude and kinetics in rat uterine strips incubated in Ca^{2+}-free PSS as well as in Na^+-free, Ca^{2+}-free PSS. However, in a K^+-free, Ca^{2+}-free PSS medium, OAC induced a response virtually identical with that observed in Ca^{2+}-free PSS, while the response to OA was highly depressed and only a small residual contraction persisted (Table 3).

All these data support the fact that the nature of the cation in OAC was potassium and also that the presence of this cation in the extracellular medium is essential for activity.

Table 3. Parameters characterizing the transient contraction induced by okadaic acid (OA, 20 μM) and its complex derivative (OAC, 20 μM) in the oestrogen-primed rat uterus in Ca^{2+}-free, Na^{+}-free and Ca^{2+}-free and K^{+} and Ca^{2+}-free solutions.

Agent	n	E_{max} (% of Ach 1mM)	Time to peak tension (min)	Duration of plateau (min)	Decrease in tone (% of OA)
Ca^{2+}-free solution					
OA	4	107.9 ± 1.1	27.2 ± 1.1	2.1 ± 1.1	85.7 ± 3.5
OAC	4	101.7 ± 2.2	23.0 ± 1.8	3.7 ± 0.5	92.8 ± 3.2
Ca^{2+}-free, Na^{+}-free solution					
OA	3	150.8 ± 16.2**	25.1 ± 3.2	3.8 ± 0.9	94.8 ± 4.0
OAC	3	186.5 ± 40.0***	30.3 ± 1.2	4.2 ± 0.8	93.3 ± 1.4
Ca^{2+}-free, K^{+}-free solution					
OA	4	6.2 ± 1.5***	28.5 ± 6.9	12.8 ± 2.4***	58.1 ± 12.5*
OAC	4	135.2 ± 5.4§	20.7 ± 2.2 †	2.9 ± 0.3†	98.0 ± 1.2†

Values are means ± s.e.m. of *n* experiments. Time to peak tension was measured from the onset of tension increase. Decrease in tone (% of maximal tension induced by the spasmogen) was measured 120 min after addition of OA or OAC. Differences from control responses in Ca2+-free solution: *P<0.05; **P<0.01; ***P<0.001, One-way ANOVA. Differences between OA- and OAC - induced effects on the same solution: †P<0.01; §P<0.001, Student's test for unpaired data.

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References and Notes

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