

Preliminary characterization of calcium binding to melanosomes isolated from amphibian oocytes

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Summary. Calcium binding to isolated melanosomes from *Xenopus laevis* and *Rana esculenta* oocytes has been assessed by equilibrium dialysis and centrifugation techniques. Calcium binding was a concentration-dependent saturable process, revealing two distinct types of binding sites. In *Xenopus* dissociation constant and maximum binding capacity were 21 μM and 0.18 nmole/oocyte for the high affinity sites ; HCl treatment destroyed the high affinity binding sites. MgCl_2 or MnCl_2 (2 mM) inhibited calcium binding to melanosomes, whereas procaine had no effect. Lanthanum (≥ 0.1 mM) was also found to be an inhibitor of high affinity sites.

Introduction.

The cytosol concentration of free Ca^{2+} in most eukariotic cells ranges from 10^{-6} to 10^{-8}M . Changes in free Ca^{2+} concentration have been proposed as critical modulators of numerous intracellular physiological and biochemical processes ; Rasmussen *et al.* (1975) have suggested that Ca^{2+} could be an important messenger for the mediation of hormone action.

The steroid hormone, progesterone, induces meiotic maturation of the full-grown amphibian oocyte (Smith, 1975). Recent studies have indicated that Ca^{2+} movements might be involved in the initiation of *Xenopus* oocyte maturation (Wasserman and Masui, 1975 ; Marot *et al.*, 1976 ; Schorderet-Slatkine *et al.*, 1976 ; Moreau *et al.*, 1976 ; O'Connor *et al.*, 1977). Evaluation of cellular Ca^{2+} metabolism is difficult because intracellular Ca^{2+} is highly compartmentalized. However, experiments in which the photoluminescent protein, aequorin, was injected into *Xenopus* oocytes have shown that free Ca^{2+} was less than 10^{-7}M and also that the mechanisms regulating its concentration were very efficient (Bellé *et al.*, 1977). The *Xenopus* oocyte must therefore contain fast Ca^{2+} sequestering equipment. An experimental approach to studying Ca^{2+} metabolism has involved the use of subcellular fractions ; Ca^{2+} interactions with subcellular fractions have shown that mitochondria (Carafoli, 1974) plasma membranes (Mc Donald *et al.*, 1976) and microsomes (Meissner *et al.*, 1973) could act as important regulators of intracellular calcium distribution. In the present

communication we demonstrate that calcium binding to isolated melanosomes from *Xenopus laevis* and *Rana esculenta* oocytes is a concentration dependent saturable process ; our results, further suggest that melanosomes could act as important regulators of intracellular Ca^{2+} in melanin containing cells.

Material and methods.

Animals. — *Xenopus laevis* from South Africa (de Rover, Holland) and *Rana esculenta* from Couetard (France) were bred and maintained under laboratory conditions.

Chemicals. — Chemicals were obtained from following sources : $^{45}\text{CaCl}_2$ (approximately 1 mCi/1,2 μmol) from CEA (France) ; collagenase (200 U/mg) from Worthington Biochemical Co ; procaine from Sigma Chemical Co, St Louiss, MO. (USA) ; pronase B grade from Calbiochem ; lanthanum chloride from Prolabo, Rhône-Poulenc (France). Equilibrium dialysis was performed using standard cellulose dialysis membranes (6 mm in diameter) from A. H. Thomas Co, Philadelphia, PA. (USA).

Oocytes preparation.

Experiments with isolated oocytes. — Specimens of *Xenopus laevis* were anesthetized with MS 222 (1 g/l) (Sandoz) Pieces of ovary were removed and the oocytes defolliculated by enzymatic treatment with collagenase (Schorderet-Slatkine and Drury, 1973). They were then equilibrated in Merriam's solution (Merriam, 1971) ; full-grown oocytes (≥ 1.2 mm diameter) were selected under stereoscopic microscope.

Cell fractionation. — Isolated oocytes or ovarian tissue were homogenized in medium A (5 ml/g of tissue) containing sucrose 0.25 M, Tris-HCl 0.05 M, KCl 75 mM, pH 7.4 with a Potter Elvehjem homogenizer. After filtration the homogenate was centrifuged at 2 000 g for 15 min. After resuspension in medium B (sucrose 2 M ; tris-HCl 0.05 M and KCl 75 mM ; potassium citrate 0.3 M, pH 7.4), the resulting pellet was centrifuged for 60 min. at 100,000 g (rotor SW-50.1). Three main fractions were obtained :

- I. A black pellet corresponding to the « melanosome » fraction ;
- II. A solid brown cap at the top of the tube representing the « membrane » fraction ;
- III. A vitellus fraction between I and II.

The melanosome fraction was washed 3 times in medium B to remove all traces of vitellogenin, and then tested for calcium binding after resuspension in medium C (Tris-HCl 0.05 M ; KCl 75 mM, pH 7.4). Before testing the vitellus fraction, the sucrose 2 M and the potassium citrate were removed by dialysis against deionized water for 24 hrs. The 2 000 g supernatant was centrifuged for 60 min. at 100,000 g ; the cytosol corresponds to the 100,000 g supernatant and the pellet to the « microsomal and mitochondrial » fraction used to test calcium binding after washing in medium A and resuspension in medium C.

Calcium-binding assay by equilibrium dialysis. — Dialysis membranes had been previously treated with 2 mM EDTA solution, followed by extensive washing with deionized water or standard buffer (medium C). After adding 1 ml of different fractions in medium C the dialysis bag was placed in an Erlenmeyer flask containing 100 ml of standard buffer in the presence of 1.0 μM to 1 mM of CaCl_2 and 0.8 μCi of $^{45}\text{CaCl}_2$. Dialysis was performed at 4 °C with stirring. Equilibrium was reached in 24 hrs. The $^{45}\text{Ca}^{2+}$ content in each compartment was determined by the use of a liquid scintillator counter.

Melanosome calcium binding assay by centrifugation. — 70 to 100 μg of melanosomal proteins suspended in 1 ml of medium C were incubated in 1 ml of standard buffer in presence of 1.0 μM to 1 mM CaCl_2 and 0.1 μCi of $^{45}\text{CaCl}_2$. After the melanosome fraction was added, the suspension was incubated for 10 min. and centrifuged at 8 500 g for 10 min. Supernatant radioactivity was assessed using a liquid scintillator counter.

Melanosome HCl hydrolysis. — Hydrolysis of the melanosome weighed sample (1 mg/ml) was carried out in 6 N HCl at 110 °C for 48 hrs. in evacuated sealed tubes. After hydrolysis, the contents were centrifuged and the pellet was washed and assayed for calcium binding.

Radioactivity counting. — Radioactivity was assayed by liquid scintillation spectrometry using 10 ml of a mixture containing PPO (5,5 g) POPOP (100 mg) in Triton X 100 (333 ml) and toluene (667 ml). Counting was carried out in a Packard Tri-carb spectrometer model 3320.

Protein assay. — Protein concentration was determined by the method of Lowry *et al.* (1951) Before the addition of Folin reagent, the proteins from the melanosome fraction were extracted with 2 p. 100 sodium dodecylsulfate at 100 °C for 2 min. (Marot *et al.*, 1977) and the melanin removed by centrifugation.

Results.

Calcium binding in subcellular fractions.

After removal of follicular cells, oocytes were homogenized and fractionated (Material and methods) ; the main fractions were assessed for total calcium binding by equilibrium dialysis (fig. 1). All fractions bound Ca^{2+} ; when saturable binding was studied high non saturable binding was found in the vitellus fraction. In the four other fractions saturable binding was observed. The melanosome fraction bound Ca^{2+} specifically with the highest binding capacity. This fraction was further analyzed, and we present here a preliminary characterization of calcium binding to purified melanosomes.

Characteristics of calcium binding to melanosomes.

Calcium binding to isolated melanosomes is a concentration dependent saturable process. Scatchard analysis of a typical binding study assayed by equilibrium dialysis is shown in figure 2. Two types of binding sites were determined. The mean K_D values obtained from a total of five determinations at 4 °C were $21 \pm 5 \mu\text{M}$ in *Xenopus*

laevis and $42 \pm 13 \mu\text{M}$ in *Rana esculenta*. In *Xenopus* binding capacity was 0.18 nmol per oocyte. No significant amount of endogenous Ca^{2+} appeared to remain bound to high affinity sites after isolation of the melanosomes; melanosomes isolated in the presence of EGTA or EDTA bound Ca^{2+} (after removal of the complexing reagents by dialysis) with a similar K_D as those reported without EDTA or EGTA in the isolation medium. This indicates that high affinity sites are entirely eluted during melanosome isolation.

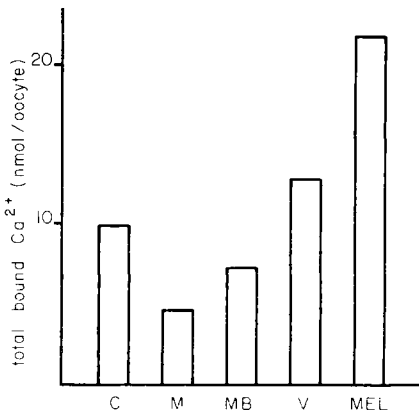


FIG. 1.

FIG. 1. — Total bound calcium in subcellular fractions. Calcium binding was determined by equilibrium in cytosol (C), mitochondrial and microsomal fraction (M), membranes (MB), vitellus (V) and melanosomal fraction (MEL) from *Xenopus* oocytes, prepared as described in materials and methods. Equivalent of 330 oocytes per ml were dialysed against 100 ml of medium C in presence of 10^{-6}M of CaCl_2 and $0,8 \mu\text{Ci}$ of $^{45}\text{CaCl}_2$ for 24 hrs.

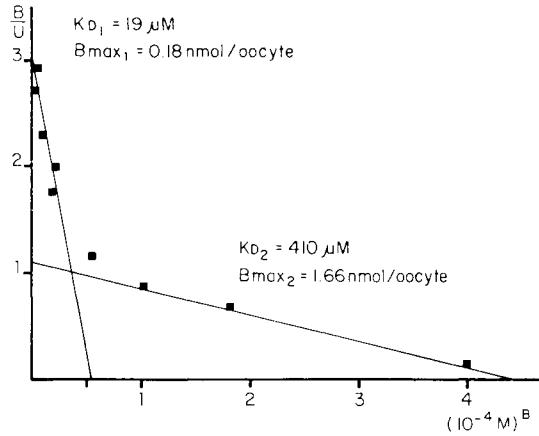


FIG. 2.

FIG. 2. — Calcium binding to melanosomes. Melanosomes were prepared as described in materials and methods. Binding was assayed by equilibrium dialysis. Protein concentration was $100 \mu\text{g}$ of protein per ml. Scatchard plot (Scatchard, 1949): B = bound Ca^{2+} ; U = free Ca^{2+} ; K_D = dissociation constant; B_{max} = maximum binding capacity.

Dialysis and centrifugation technique yield similar affinity constants. The centrifugation technique was used in subsequent studies since it was easier and more rapid.

As measured by centrifugation, calcium binding to melanosomes reached a steady state in 10 min. at all calcium concentrations studied.

The calcium binding was linear between 10 and $200 \mu\text{g}$ of melanosomal proteins in each sample. Above $200 \mu\text{g}$ the binding became non-linear. All binding assays contained between 50 to $100 \mu\text{g}$ of proteins.

In order to know if Ca^{2+} bound to melanoproteins or to the melanin polymer, proteins were removed by 6 N HCl hydrolysis. Calcium binding to the melanin was assessed by equilibrium dialysis, but high affinity binding was never detected, indicating that binding sites were either removed by the HCl treatment or were destroyed. Treatment of melanosomes with pronase ($0,3 \text{ mg/ml}$) for 12 hrs at 4°C slightly increased calcium binding to melanosomes.

All standard assays were performed at pH 7.4 ; calcium binding steadily increased from pH 6.0 to pH 9.0 (fig. 3).

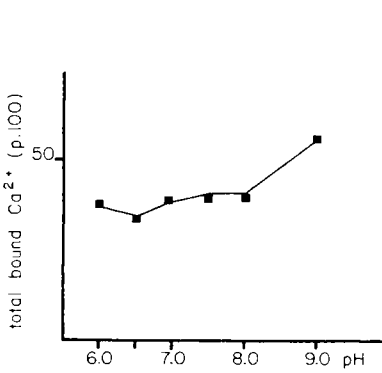


FIG. 3.

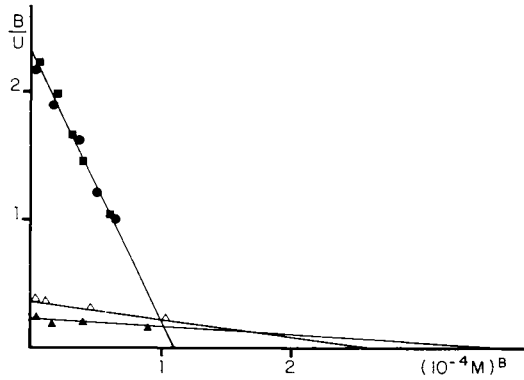


FIG. 4.

FIG 3. — Effect of pH on calcium binding to melanosomes. Centrifugation assays were performed in the presence of 20 μM of CaCl_2 , as described in materials and methods. Binding is expressed as the percentage of bound calcium/total calcium in the medium.

FIG. 4. — Effect of lanthanum chloride on calcium binding to melanosomes. Calcium binding was assayed by equilibrium dialysis in the presence of various amounts of Ca^{2+} (1 μM to 1 mM) and of three La^{3+} concentrations: 10^{-6}M (■—■) 10^{-4}M (Δ — Δ) and 10^{-2}M (\blacktriangle — \blacktriangle). Control without La^{3+} (○—○). Protein concentration was 50 μg per ml. Scatchard plot (15) : B = bound Ca^{2+} ; U = free Ca^{2+} .

Effect of different ions and procaine.

When KCl concentration was less than 100 mM, calcium binding was unmodified. Above that concentration, binding was inhibited ; 50 p. 100 of the original binding level was reached at 2 M KCl.

Ca^{2+} binding in the presence of 2 mM Mn^{2+} was tested ; both divalent cations inhibited calcium binding. About 50 p. 100 inhibition was achieved at 2 mM of Mn^{2+} or Mg^{2+} .

Lanthanum was also found to be an inhibitor of calcium binding (fig. 4). A La^{3+} concentration of 0.1 mM totally inhibited calcium binding to melanosomes.

Procaine 0.5 mM to 5 mM did not inhibit calcium binding to melanosomes.

Discussion.

The binding of calcium to melanosomes is a saturable process which exhibits two distinct types of binding sites. The high affinity dissociation constant K_D was found to be about 20 μM in both *Xenopus* and *Rana*. This value is comparable with those reported for Ca^{2+} binding to plasma membranes, microsomes or to numerous calcium binding proteins (Mc Donald *et al.*, 1976 ; Kretsinger, 1976).

Melanosomes are complex subcellular organelles surrounded by a membrane composed of insoluble melanin polymer and firmly bound proteins in unequal pro-

portions. A preliminary characterization of amphibian melanoproteins was achieved after SDS extraction (Marot *et al.*, 1977), at least twenty proteins were resolved by polyacrylamide gel electrophoresis. In order to know the localisation of Ca^{2+} binding sites, melanoproteins were extracted by SDS (data not given), and it was found that the binding sites were not extracted in these conditions. As reported by Marot *et al.* (1977), only HCl (6 N) hydrolysis for 20 hrs. removes all melanosomal proteins and abolishes Ca^{2+} binding to melanin. It was reported that synthetic melanin only binds Ca^{2+} very slightly (Potts and Au, 1976). This suggests Ca^{2+} binding at the melanoprotein level and, to our knowledge, it is the first report attributing a biochemical function to melano-proteins.

The finding that Mg^{2+} and Mn^{2+} at high concentration (2 mM) inhibit Ca^{2+} binding to melanosomes is of interest ; it was demonstrated that the ionophore A 23187 in the presence of Mg^{2+} (10 mM) (Wasserman and Masui, 1975) or Mn^{2+} (10 mM) (unpublished results) triggered *Xenopus* oocyte maturation in the absence of hormonal stimulation. These divalent cations could induce a Ca^{2+} release by inhibiting calcium binding to melanosomes. However, the conclusions are ambiguous regarding the biological significance of Ca^{2+} binding to oocyte melanosomes, particularly during hormonally induced maturation.

Total calcium concentration in the whole oocyte ranges from 1 to 10 mM ; Ca^{2+} binding capacity of melanosomes at high affinity sites represents between 2 to 20 p. 100 of total Ca^{2+} . Recent experiments using microprobe analysis (unpublished results) confirm that melanosome Ca^{2+} content is high. It is suggested that melanosomes may play a role in the regulation of calcium metabolism in amphibian oocytes.

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Résumé. La fraction des mélanosomes a été isolée par ultracentrifugation à partir des ovocytes de *Xenopus laevis* et de *Rana esculenta*. La fixation du Ca^{2+} sur les mélanosomes a été étudiée par dialyse à l'équilibre et par centrifugation. La liaison du Ca^{2+} aux mélanosomes est un phénomène saturable. Chez le Xénope, la constante de dissociation est de 21 μM ; la capacité maximum de liaison pour les sites de haute affinité est de 0,18 nmole/ovocyte. Les sites de haute affinité pour le Ca^{2+} sont détruits après traitement par HCl (6 N). MgCl_2 ou MnCl_2 à la concentration de 2 mM inhibe la fixation du Ca^{2+} tandis que la procaine n'a pas d'effet sur la liaison du Ca^{2+} aux mélanosomes. Le lanthane à une concentration $\geq 0,1$ mM est un inhibiteur des sites de haute affinité.

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