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Original article

Ca^{2+} as a messenger of dorsal–ventral polarity formation in frog *(Rana temporaria)* eggs*

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Summary — Three methods of Ca^{2+} administration were used to influence the location of the grey crescent and the dorsal lip of blastopore in *R temporaria* eggs, *ie* a Ca^{2+} microinjection into the subcortical cytoplasm, egg pricking in high Ca^{2+} solutions and Ca^{2+} inophore A23187 microinjection and application. The treatments all induced grey crescent and dorsal lip of blastopore formation near the Ca^{2+} administration site. Inositol trisphosphate injections gave similar results. Colchicine injections into the eggs inhibited the appearance of both natural and Ca^{2+}-induced grey crescents.

egg polarity / grey crescent / dorsal lip / blastopore / calcium / inositol trisphosphate

Résumé — Le calcium (Ca^{2+}), un messager pour l'induction de la polarité dorso-ventrale dans les œufs de grenouille *(Rana temporaria)*. *Trois méthodes d'administration de Ca^{2+} ont été utilisées pour agir sur la localisation du croissant gris et de la lèvre dorsale du blastopore dans les œufs de* R temporaria*: microinjection de calcium dans le cytoplasme subcortical, piqûre de l'œuf dans les solutions riches en Ca^{2+}, microinjection et application de Ca^{2+} ionophore A23187. Les traitements ont tous induit la formation du croissant gris et de la lèvre dorsale du blastopore près du lieu d'administration du Ca^{2+}. L'injection de triphosphate d'inositol a donné les mêmes résultats. L'injection de colchicine dans les œufs a bloqué la formation du croissant gris, aussi bien naturelle qu'induite par le Ca^{2+}.*

polarité / croissant gris / lèvre supérieure / blastopore / calcium / triphosphate d'inositol

INTRODUCTION

Dorsal–ventral (D–V) polarity in amphibian eggs is specified by fertilization which triggers a number of mechano-chemical processes resulting in egg structure rearrangements (Wakahara, 1989). A grey crescent (GC) appearing on the D side of the frog egg serves as a marker of these rearrangements. It has been demonstrated that the dorsal side of the egg or GC, in particular, is formed opposite the sperm entrance site (SES) as a result of cytoplasmic rotation relative to the egg surface (Ancel and Vintemberger, 1948; Vin-

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cent *et al*, 1986). However, the question of how the direction of subcortical rotation and localization of the GC is determined has not yet been resolved.

In our previous paper we have demonstrated the possibility of modifying the GC and the D–V axis position by means of local Ca^{2+} administration into *R temporaria* eggs (Bozhkova *et al*, 1990). Ca^{2+} was injected into both activated (during activation) and fertilized eggs (10–20 min after insemination). In both cases, Ca^{2+} destroyed the connection between the SES and the GC, and the formation of the GC and dorsal lip of a blastopore (DLB) occurred predominantly near the injection site. In the present study we demonstrate the effects of Ca^{2+} ionophore A23187 and inositol trisphosphate ($InsP_3$). These substances trigger the intracellular release of Ca^{2+} in a variety of cells including unfertilized eggs (Busa *et al*, 1985; Berridge, 1988; Iwamatsu *et al*, 1988). An attempt was also made to find the target of the Ca^{2+} effect.

MATERIALS AND METHODS

Procedures for the collection and fertilization of *Rana temporaria* eggs have been described by Bozhkova *et al* (1990). The eggs were oriented with the animal–vegetal axis verticale before insemination. The basal solution was artificial pond water (APW). Other solutions were prepared from the basal solution supplemented with various salts or other compounds. All experiments were carried out at 18–19 °C. Procedures for injections have been described by Bozhkova *et al* (1990) as well as for the determination of GC and DLB localization relative to the site of treatment (fig 1).

Egg treatments

The following solutions were injected into the eggs through micropipettes: $CaCl_2$ (20 mM), KCl (30 mM), NaCl (1–5 M), colchicine (Merck) (6 mg/ml), $InsP_3$ (Sigma) (1 mM), A23187 (Calbiochem) (20 μM), DMSO (Serva) (0.2%). All substances were added to the solution which contained 5 mM HEPES and 0.2 mM fluorescein (pH 7). The A23187 solution contained 0.2% DMSO. The injections were made in the subcortical region of the egg cytoplasm near the boundary between the pigmented and unpigmented domains (Bozhkova *et al*, 1990). The injected volume was 2–10 nl for Ca^{2+}, K^+ and Na^+, 8–16 nl for colchicine and A23187 and 8 nl for $InsP_3$. The injections were made at various times between fertilization and GC formation. A23187 was microinjected into the frog eggs at a concentration which induced intracellular Ca^{2+}-release (Iwamatsu *et al*, 1988). Another technique to maintain high local Ca^{2+} concentration was to prick the egg with a thin glass microelectrode in APW + 10 or 20 mM $CaCl_2$. Ca^{2+} ionophore A231897 was also applied externally and locally. It was injected through the micropipettes (20–40 nl) into the jelly coats near the egg surface in the equatorial region of the egg. The injected solution contained 1 mM A23187, 5 mM HEPES, 0.2 mM fluorescein and 10% DMSO (pH 7). The applications were performed once during the 15–20 min after insemination or twice during the 15–20 and 30–50 min after insemination in the same place. As a control, the same solutions without Ca^{2+} ionophore were applied.

RESULTS

The effect of Ca^{2+}, Ca^{2+} ionophore A23187 and $InsP_3$ on D–V polarity

Observations of the GC and DLB in frog eggs have revealed that they appeared randomly relative to the site of pricking in APW + 20 mM NaCl (table I). The DLB percentage ratio was 33:40:27 (sector or prick: lateral sectors: sector opposite to a pricked sector). It remained almost unchanged and did not differ from the random distribution if control solutions (HEPES or KCl) were injected into the eggs (fig 1, table I). However, administration of Ca^{2+} by microinjection and by egg

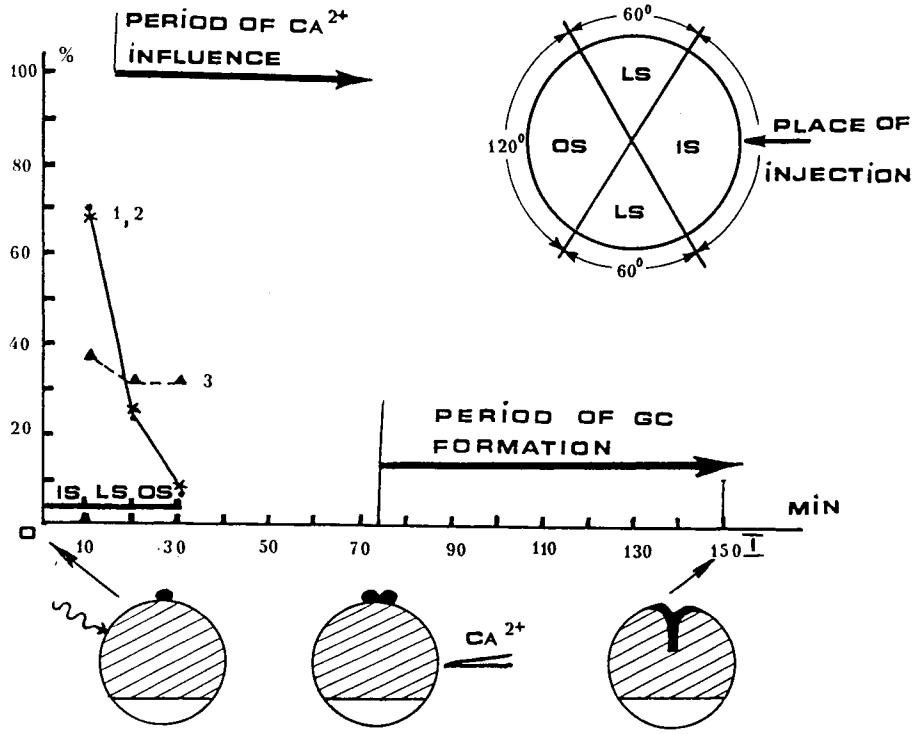


Fig 1. Relationship between the period of the grey crescent formation and the time at which the Ca^{2+} influence on this process was detected. Abscissa: time from insemination to the first cleavage (at 19° C) (min). On the left: proportion (%) of eggs with grey crescents forming in various sectors relative to the treatment site (1, Ca^{2+} microinjections into eggs; 2, egg pricking in APW + 10 or 20 mM $CaCl_2$; 3, K^+ microinjections into eggs). On the right: diagram showing the determination of grey crescent location relative to the place of Ca^{2+} injection. Abbreviations: is = injected sector; ls= lateral sectors; os = sector opposite to the injected sector.

pricking in high Ca^{2+} solutions within 10–20 min after insemination induced GC and DLB formation near the injection site in 60–90% of cases.

To check the specificity of the Ca^{2+} effect, Na^+ was injected in concentrations which resulted in a considerable relative increase of this ion in the egg cytoplasm. The highest concentration of the injected NaCl which did not disturb cleavage and the following development was 1 M. When 1 M NaCl was injected, a weak influence on GC and DLB localization was observed (table I), but the difference between the NaCl and the control solutions treatments (HEPES or KCl) was not significant (P = 0.60 or P = 0.53 respectively), while the difference between NaCl and $CaCl_2$-injected eggs was significant (P = 0.0017; χ^2-test).

Table I. Ca^{2+} influence on dorsal-ventral polarity.

Method	*Marker of D–V polarity*	*Solution*	*Localization of DLB relative to injection (%)*			*No of checked embryos and spawning*	*P*****
			is	*ls*	*os****		
Injection	DLB	$CaCl_2$	73	25	2	48,3	0
	DLB	KCl	40	35	24	62,3	0.28
	DLB	NaCl	48	27	25	81,3	0.02
	GC	A23187*	57	26	16	267,6	0
	GC	DMSO	37	34	29	70,3	0.67
	DLB	$InsP_3$	61	31	8	90,5	0
	DLB	HEPES	41	31	28	89,2	0.25
Prick	DLB	$CaCl_2$	62	32	7	72,5	0
	DLB	NaCl	33	40	27	92,5	0.31
Application	DLB	A23187**	43	43	14	143,5	0
	DLB	DMSO	32	35	33	53,2	0.96

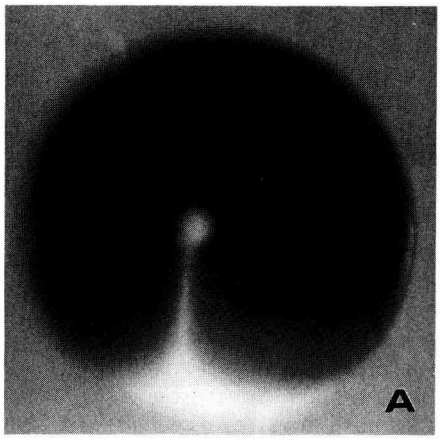
* In these experiments the orientation of the D–V axis was checked mainly by the position of the GC because gastrulation was sometimes disturbed by A23187 injection. ** The locally applied ionophore induced the activation of unfertilized eggs. *** is = injected sector; ls = sectors lateral to injected sector; os = sector opposite to the injected sector. **** *P* = comparison of the obtained data with a random distribution (χ^2 test).

The introduction of $InsP_3$ and A23187 by injection within 10–15 min after insemination resulted in preferential GC and DLB formation in the injected region (table I). The application of Ca^{2+} ionophore had a similar though less pronounced effect (table I). GC and DLB location in sectors was not random (as seen from the χ^2 criterion) and they were mainly found in the sector of application and in the lateral sectors.

Local motility of the wound surface and GC formation

A Ca^{2+}-induced GC was formed at the same time as the control, but was somewhat different from the natural crescent: it was lighter in colour and larger in size. As a rule, the GC region contained a wound indicating the site of Ca^{2+} administration. When media with a high Ca^{2+} content were used (Bozhkova *et al*, 1990), the injection wound was located on the top of the GC with a light band extending from it down to a boundary between the pigmented and unpigmented areas (fig 2b). Such a band developed earlier than GC as a result of local wound motility from the site of the Ca^{2+} prick to the A pole (fig 2a).

The local surface motility of the wound correlated positively with a probability of GC appearance in the injection sector. Each motile wound was later included into the area of the forming GC, as if predicting it beforehand. As a result, the proportion of eggs with motile wounds corresponded to the number of eggs with the GC formed in the injection sector (fig 3).



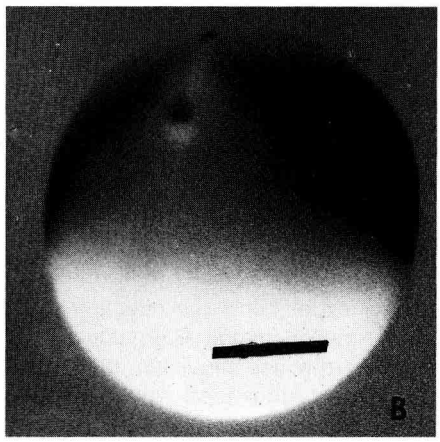


Fig 2. Stages of Ca^{2+}-induced GC formation in APW + 10 or 20 mM $CaCl_2$: (A), displacement of wound from the injection site to the A pole (60 min after fertilization); (B), Ca^{2+}-induced GC (1.5–2 h after fertilization). Bar, 0.5 mm.

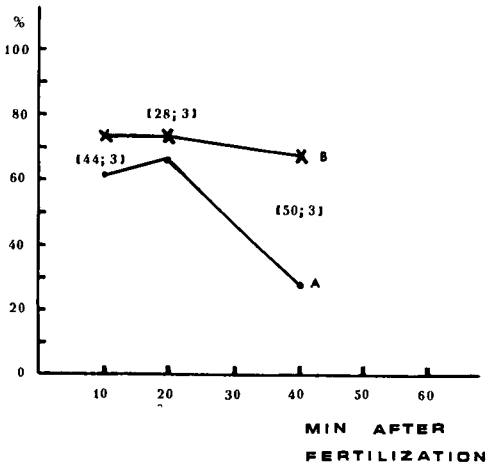


Fig 3. Proportion (%) of eggs with motile wounds (A) and eggs with a grey crescent forming in the injected sector (B). Abscissa: time of egg pricking in APW + 10 or 20 mM $CaCl_2$.

The question is whether this premature shifting of the surface near the injection site is necessary for the formation of the Ca^{2+}-induced GC. We studied the behavior of the wound under various conditions and concluded that this was obviously not the case. Firstly, in experiments with intracellular injections of Ca^{2+}, when APW or APW + 20 mM NaCl were used as the external solutions, the wound remained immobile though Ca^{2+} produced a clear orienting effect (table I). Secondly, the ability of the wound to move depended on the time of pricking: it was maximal when the prick was made 10–20 min after insemination, and had almost completely disappeared by 40 min (fig 3). In spite of this, the orienting effect persisted to a considerable degree in both cases (fig 3).

Colchicine treatment

The role of cytoskeleton microtubules was studied to determine which cytoplasmic structures were responsible for the directed movement of the surface during the Ca^{2+}-induced GC formation. Colchicine was injected within 15–20 min after insemi-

nation and 25–5 min prior to the onset of the first signs of GC formation. In all 79 cases of natural development, GC formation either did not start at all (72% of cases) or ceased at the initial stage (28% of cases). A similar result was described for *Xenopus laevis* (Vincent *et al,* 1987). Colchicine injections at the same stages of development also inhibited the formation of Ca^{2+}-induced GC generated by egg pricking in APW + 10 or 20 mM $CaCl_2$ (49 eggs from 3 spawnings).

DISCUSSION AND CONCLUSION

Thus, the local administration of Ca^{2+} by 3 different methods (direct injection into the cytoplasm, pricking the egg in high Ca^{2+} solutions, Ca^{2+} ionophore A23187 injection and application) can specify the location of the GC and the DLB in *R temporaria* eggs, overriding the normal axis specification mechanism. The results of $InsP_3$ injections were similar to those of Ca^{2+} injections. The similarity of the effect of local Ca^{2+} administration (injected Ca^{2+} concentration up to 10–20 mM) on the one hand and the effect of Ca^{2+} ionophore A23187 and $InsP_3$ on the other could be explained by a great (millimolar) concentration of the total calcium sequestered in the egg stores (Gillot *et al,* 1989). As a result, free Ca^{2+} concentration in the egg (initial level near 1 μM; Busa and Nuccitelli, 1985) would be ≈ 10 times as high after injections. There was no damage to embryonic development after Ca^{2+} injection, probably because of Ca^{2+} sequestration in the intracellular stores. A rise of Na^+ after the 5 M NaCl injection, up to a level 2–3 times higher than in the control eggs (initial level near 10 mM; Gillespie, 1983), resulted in the inhibition of egg cleavage. The use of a non-lethal dose (1 M NaCl) for injection did not result in the reorientation of the D–V axis as it did after Ca^{2+} administration. The weak effect of the Na^+ injection (table I) could be explained by the influence of small ions on Ca^{2+} efflux from the intracellular stores (Muallem *et al,* 1985).

Is the Ca^{2+} effect on the formation of GC and DLB an exclusively experimental phenomenon or does Ca^{2+} take part in normal GC formation? It is known that a temporal increase in the Ca^{2+} level along the subcortical layer of the cytoplasm is a specific feature of fertilization (Busa and Nuccitelli, 1985; Speksnijder *et al,* 1986). An increase in the level of $InsP_3$ has been also recorded in eggs after fertilization (Kamel *et al,* 1985; Ciapa and Whitaker, 1986). The Ca^{2+} release can be mimicked by $InsP_3$ injection into eggs (Whitaker and Irvine, 1984; Busa *et al,* 1985). Perhaps during normal development the same processes provide an increasing Ca^{2+} gradient from the SES to the opposite side of the egg where the dorsal side would form. The hypothesis of a Ca^{2+} effect on polarity of ascidian eggs has also been reported (Jeffery, 1982; Speksnijder *et al,* 1990).

In our experiments the direction of the surface rotation during Ca^{2+}-induced GC formation may be predicted beforehand from the direction of local wound displacement (if there is Ca^{2+} in the medium and the prick is made before 40 min after fertilization). Despite the fact that wound displacement is not a necessary condition for surface rotation, it is indicative of a possible predetermination of rotation direction before GC appearance.

Spatial organization of the egg microtubules is a possible mechanism of surface movement orientation during naturally determined GC formation (Elinson and Rowning, 1988). Vincent *et al* (1987) have shown that GC formation ceases within several minutes after colchicine injection. Our finding has confirmed this observation

with Ca^{2+}-induced GC. However, at present it is still unclear as to how Ca^{2+} may induce spatial organization of subcellular structures.

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