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Activation of Sea Urchin Eggs by 1-fluoro-2, 4-dinitrobenzene

By

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(With 5 Text-figures)

Introduction

In course of studies on the probable relationship between the energy source of cortical reaction in sea urchin egg and that of muscle contraction (Cain and Davies, 1962), artificial induction of the cortical reaction by 1-fluoro-2, 4-dinitrobenzene (FDNB), an inhibitor of creatine kinase, was found. It is well known, on the other hand, that Ca^{2+} ionophore, A23187, induces the cortical reaction in sea urchin eggs (Chambers *et al.*, 1974; Steinhardt and Epel, 1974).

The present paper deals with the mode of activation of FDNB, active part of the molecule and the difference between the action site of FDNB and that of Ca^{2+} ionophore, ionomycin in the cascade of the cortical reaction.

Materials and Methods

Materials used were gametes of the following three species of the sea urchins: *Strongylocentrotus intermedius*, *Strongylocentrotus nudus* and *Hemicentrotus pulcherrimus*. The data obtained with the first species was mainly cited in the present paper.

Handling of the sea urchin gametes

Eggs were obtained by pouring 0.5 M KCl into the opened body cavity and washed with artificial sea water (ASW) three times before use. Adequate precautions were taken against contamination with sperm. The ionic composition of ASW was as follows (mM): NaCl, 458; KCl, 9.7; CaCl_2 , 10.3; MgCl_2 , 48.5; Tris, 10; (pH 8.2). Spermatozoa of the homologous species were obtained by cutting

ripe testis removed from dissected male. Sperm concentration was expressed by dilution from the original semen ("dry" sperm). Batches showing over 98% fertility were used for experiments. All experiments were carried out at 20°C.

Treatment of the eggs with chemicals

1) *FDNB and CDNB*

1-fluoro-2, 4-dinitrobenzene (FDNB), fluorobenzene and 1-chloro-2, 4-dinitrobenzene (CDNB) were dissolved in dimethylsulfoxide (DMSO) to 0.1 M respectively and stored at 4°C until use. To 2 ml of various concentrations of FDNB in ASW, 10 μ l of eggs was added and after 5 minute incubation, the number of the eggs with fertilization envelope was counted by a light microscope. Percentage of eggs with fertilization envelope is shown as that of FDNB-induced activation. Eggs treated with fluorobenzene or CDNB were also observed at the same conditions as applied with FDNB.

2) *SH-group*

Treatment of the eggs with FDNB in the presence of L-cysteine was also performed. To 2 ml of various concentrations of L-cysteine in ASW, 2 μ l of 0.1 M FDNB was added and after 10 minute incubation, 10 μ l of eggs was added. After 5 minutes, the number of eggs with fertilization envelope was counted. At the same time, fertilization rate of the eggs by 10^{-5} dilution of dry sperm was counted in the presence of respective concentrations of L-cysteine.

3) *Blocking of intracellular SH-group*

Eggs pretreated with various concentrations of iodoacetamide, a SH-blocking reagent, were rapidly washed two times in ASW after 10 minute incubation. To 2 ml of 0.1 mM FDNB, 10 μ l of these eggs were added. After 5 minutes, the number of eggs with fertilization envelope was counted. At the same time, fertilization rate of iodoacetamide-pretreated eggs by 10^{-5} dilution of dry sperm were counted.

4) *TMB-8*

Eggs pretreated with various concentrations of 3, 4, 5-trimethoxybenzoic acid 8-diethylamino octyl ester (TMB-8), an inhibitor of intracellular Ca^{2+} mobilization (Clapper and Lee, 1985; Stapleton *et al.*, 1985), were rapidly washed two times in ASW after 10 minute incubation. To 2 ml of 0.1 mM FDNB or 5 μ M ionomycin, 10 μ l of these eggs were added respectively. After 5 minutes, the rate of activation of each group was counted.

5) *Cytochalasin B*

Cytochalasin B was dissolved in DMSO to 3 mM and ionomycin in ethanol to 5 mM, both stored at $-20^{\circ}C$ and diluted before use. Eggs treated with 3 μ M cytochalasin B, an inhibitor of actin polymerization, were rapidly washed two times in ASW after various incubation times. To 2 ml of 0.1 mM FDNB or 5 μ M ionomycin, 10 μ l of these eggs was added respectively. After 5 minutes, the number of eggs with fertilization envelope in each group was counted.

Chemicals

FDNB, CDNB, fluorobenzene, sodium fluoride and L-cysteine were purchased from Kishida Chemical Co., LTD. (Osaka, Japan). Iodoacetamide and DMSO (dimethyl sulfoxide) were the products of Nakarai Chemicals, LTD. (Kyoto, Japan). TMB-8 and cytochalasin B were the products of Sigma Chemical (U.S.A.). Ionomycin was from Hoechst (La Jolla, CA).

Results

1) FDNB causes cortical reaction.

When unfertilized eggs of the sea urchin, *Strongylocentrotus intermedius*, were immersed in more than 100 μM FDNB dissolved in ASW, fertilization envelope was formed in 2 to 5 minutes in more than 98% of eggs of all batches examined (Fig. 1). Similar results were obtained with the eggs of *Strongylocentrotus nudus* and *Hemicentrotus pulcherrimus*. No cytolysis was observed within 2 hours in the presence of external FDNB at 100 μM , so that this concentration was used in the following experiments. DMSO (0.1%), in which FDNB was dissolved, did not cause fertilization envelope formation. In the concentration below 50 μM , the number of eggs with fully elevated envelope gradually decreased (Fig. 1). The rate of egg-activation by fluorobenzene, devoid of two nitro radicals of FDNB, was less than 30% at 100 μM .

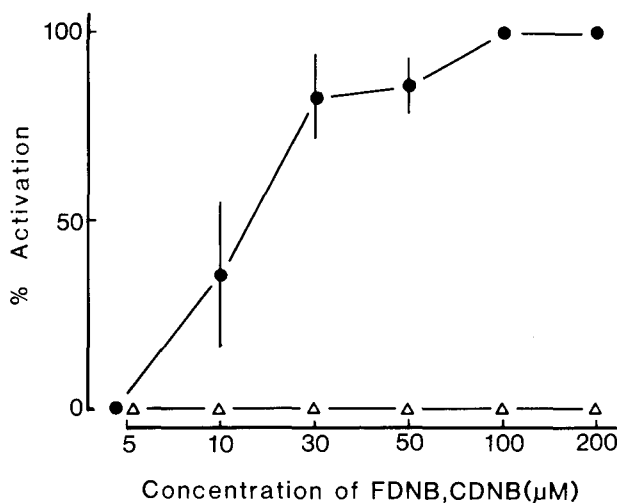


Fig. 1. Effect of FDNB (●) on activation of sea urchin eggs. To various concentrations of FDNB in ASW, the eggs were added at 20°C. Percentages of eggs with fertilization envelope after 5 minute incubation are shown. Ineffectiveness of CDNB (△) is also shown at respective concentrations applied for FDNB. Values shown are means \pm standard errors of three experiments with different females.

When the eggs were treated with CDNB, the derivative substituted chlorine for fluorine of FDNB, at the same concentrations of FDNB, fertilization envelope formation was not observed at all in every concentration examined (Fig. 1). It was assumed, therefore, that fluorine contained in FDNB participates in the egg-activation.

2) *SH-group in external medium inhibits FDNB-induced activation.*

To investigate the mechanism of egg activation by fluorine, the eggs were kept in ASW containing 1 mM or 5 mM sodium fluoride for 10 minutes. Fertilization envelope formation was not observed. Extracellular F^- has not the activating effect on eggs. This was confirmed by the other kind of experiment. FDNB is known to release F^- in consequence of the reaction with SH-groups (Wallenfels and Streffer, 1966; Gold, 1968). When the eggs were treated with the medium containing both FDNB and L-cysteine, activation was significantly depressed. The number of eggs with fully elevated envelope reciprocally decreased as the concentration of L-cysteine increased from 0.05 mM to 1 mM. L-cysteine itself was not injurious for the eggs. In the presence of external L-cysteine at any concentration examined, fertilization envelopes were formed by sperm in more than 98% eggs (Fig. 2). These results suggest that the effect of FDNB is exerted

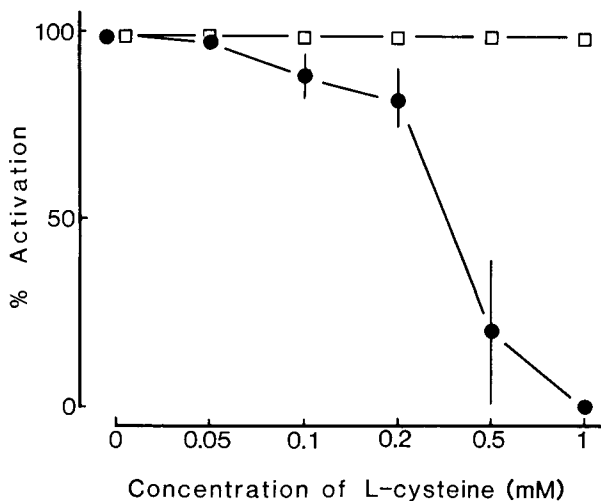


Fig. 2. Inhibitory effect of L-cysteine on FDNB-induced activation. To various concentrations of L-cysteine in ASW, FDNB (●) was added and after 10 minute incubation the eggs were added. Percentages of eggs with fertilization envelope are shown. Count was performed at 5 minutes after the addition of the eggs. Fertilizability (□) of the eggs in the presence of L-cysteine is also shown at respective concentrations. Values shown are means \pm standard errors of the results of three experiments with different females.

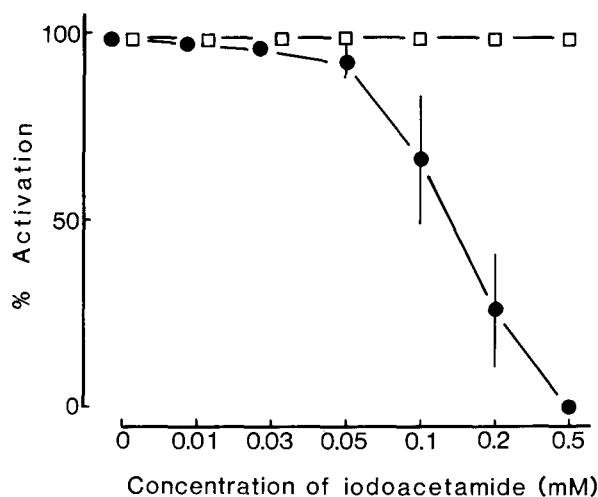


Fig. 3. Inhibitory effect of SH-blocking reagent on FDNB-induced activation. Eggs treated with various concentrations of iodoacetamide were rapidly washed two times in ASW after 10 minute incubation and added to 0.1 mM FDNB (●). Percentages of eggs with fertilization envelope are shown. Count was performed at 5 minutes after the addition of the eggs. Fertilizability (□) of iodoacetamide-pretreated eggs was also shown at the respective concentrations of iodoacetamide. Values shown are means \pm standard errors of the results of five experiments with different females.

by intracellularly released F^- .

3) *Blocking of intracellular SH-group inhibits FDNB-induced activation.*

If F^- is released in consequence of the reaction of FDNB entered into the egg with intracellular SH-groups and stimulates some component(s) in the cascade of cortical reaction, the blocking of intracellular SH-groups will result in inhibition of FDNB-induced activation. To test this hypothesis, the eggs were pretreated with iodoacetamide. As shown in Fig. 3, the eggs pretreated with iodoacetamide at 0.5 mM or more were not activated by FDNB. The inhibition was concentration-dependent above 0.01 mM till 0.2 mM. When iodoacetamide-pretreated eggs were inseminated by 10^{-5} dilution of dry sperm, fertilization envelope was formed in more than 98% eggs (Fig. 3). Injury of the eggs by iodoacetamide is unlikely in the present conditions.

4) *TMB-8 inhibits FDNB-induced activation.*

Ca^{2+} ionophore, A23187, which is known to make cytosolic Ca^{2+} level high enough to cause egg-activation (Steinhardt and Epel, 1974; Chambers *et al.*, 1974) is widely used as the activating reagent. Comparison between the action of Ca^{2+} ionophore and that of FDNB was performed to infer the step(s) FDNB acts in the cascade of the cortical reaction. In this experiment, the eggs were pretreat-

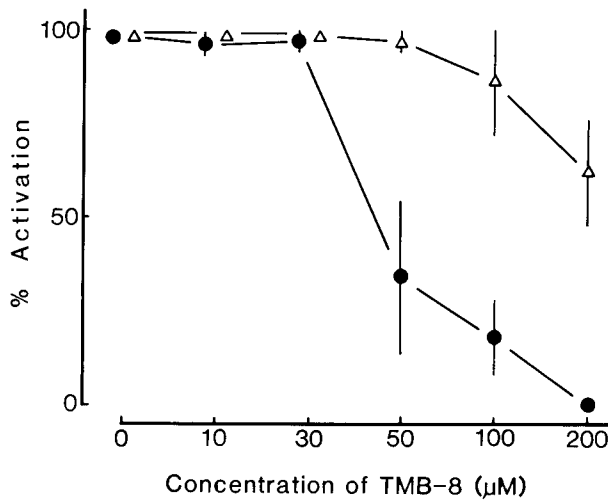


Fig. 4. Difference between the action of FDNB and that of ionomycin. Eggs treated with various concentrations of TMB-8 were rapidly washed two times in ASW after 10 minute incubation and added to 0.1 mM FDNB (●) or 5 μ M ionomycin (Δ). Percentages of the eggs with fertilization envelope are shown at respective concentrations of TMB-8. Count was performed at 5 minutes after the addition of the eggs. Values shown are means \pm standard errors of the results of four experiments with different females.

ed with TMB-8 and then with ionomycin, a Ca^{2+} ionophore (Liu and Hermann, 1978) or with FDNB. As shown in Fig. 4, while more than 95% of eggs pretreated with 50 μ M TMB-8 was activated by 5 μ M ionomycin, only about 35% of the pretreated eggs were activated by FDNB. In case of the eggs pretreated with 200 μ M TMB-8, activation by FDNB was entirely inhibited. On the contrary, ionomycin still caused activation in more than 60% of the eggs (Fig. 4).

5) *Cytochalasin B inhibits both FDNB- and ionomycin-induced activation.*

Reagents inhibiting ionomycin-induced activation were searched, and it was found that cytochalasin B makes the eggs insensitive to both FDNB and ionomycin (Fig. 5). Sharp decrease of activation rate was observed by more than 20 minute pretreatment with 3 μ M cytochalasin B, and complete loss of sensitivity was seen by 50 minutes.

Bringing this result together with that obtained with cytochalasin B, it may be suggested that FDNB affects the step(s) which exist(s) before that Ca^{2+} ionophore does in the cascade of the cortical reaction.

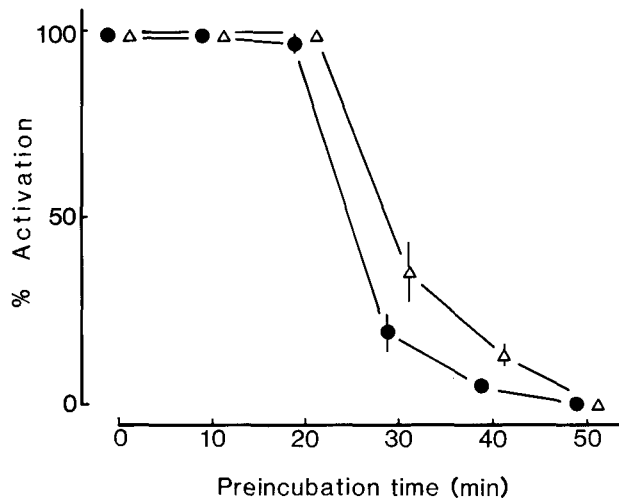


Fig. 5. Inhibitory effect of cytochalasin B on FDNB- or ionomycin-induced activation. Eggs treated with cytochalasin B ($3 \mu\text{M}$) for various preincubation times were rapidly washed two times in ASW and added to 0.1 mM FDNB (\bullet) or $5 \mu\text{M}$ ionomycin (Δ). Percentages of the eggs with fertilization envelope are shown at various preincubation times in cytochalasin B. Count was performed at 5 minutes after the addition of the eggs. Values shown are means \pm standard errors of the results of three experiments with different females.

Discussion

The present study shows that FDNB artificially activates sea urchin eggs by forming the fertilization envelope. CDNB, the derivative substituted chlorine for fluorine of FDNB, was not effective. FDNB is known to release F^- by reaction with SH-groups (Wallenfels and Streffer, 1966; Gold, 1968). By a similar manner, CDNB releases Cl^- instead of F^- (Gold, 1968). It may be suggested, therefore, that the activation by FDNB is caused not by blocking SH-groups but by releasing fluorine. As described in the preceding section, the activation was not observed in ASW containing sodium fluoride, and the effect of FDNB was cancelled by L-cysteine. These results suggest that extracellular F^- is not effective for the activation. If the effect of FDNB depends upon intracellular F^- , the blocking of intracellular SH-groups should prevent FDNB-induced activation. This was the case. The eggs pretreated with iodoacetamide became insensitive to FDNB. It may be concluded that intracellular F^- released in consequence of the reaction of FDNB entered into the egg with intracellular SH-groups stimulates some step(s) in the cascade of the cortical reaction.

FDNB is a benzene compound. According to Ishikawa (1954), benzene

compounds having methyl-, phenolic hydroxyl-, pyrrol- or aldehyde-radical are effective for the egg activation so long as nitro- or carboxyl-radicals are not present. He considers that the nitro radical cancels the effectiveness of the compounds. For example, 2,4-dinitrophenol in which phenol has two nitro radicals is ineffective. In the present study, however, it has been shown that FDNB, which has two nitro radicals, is superior to fluorobenzene lacking this radical as to the egg activation. Difference of membrane permeability should not be ignored in this kind of works. Nitro-radicals may make fluorobenzene more permeable to the egg.

It was found that Ca^{2+} ionophore, A23187, activates sea urchin eggs even in Ca^{2+} free ASW (Chambers *et al.*, 1974; Steinhardt and Epel, 1974). It is widely recognized that fertilization is accompanied by an increase in cytosolic free Ca^{2+} level (Steinhardt *et al.*, 1977; Eisen *et al.*, 1984). It is considered that Ca^{2+} ionophore enters into the egg and releases Ca^{2+} from intracellular storages to make cytosolic Ca^{2+} level high enough to cause the egg activation.

The results obtained in the present study reveals that the mode or the site of activation by FDNB was different from that of ionomycin. While the former did not activate the eggs pretreated with TMB-8, the latter did. The condition in which the effect of ionomycin was cancelled by the pretreatment with cytochalasin B also cancelled the effect of FDNB. It may be concluded that the action site of FDNB in the cascade of cortical reaction exists before that of Ca^{2+} ionophore.

FDNB caused activation also of both *Strongylocentrotus nudus* and *Hemicentrotus pulcherrimus* eggs by a similar manner. It is an useful probe for fundamental comprehension of the cortical reaction in sea urchin eggs.

Summary

When the eggs of the sea urchin, *Strongylocentrotus intermedius*, were treated with 1-fluoro-2, 4-dinitrobenzene (FDNB), they were activated forming the fertilization envelope in 2 to 5 minutes. 1-chloro-2, 4-dinitrobenzene (CDNB), another derivative of dinitrobenzene substituted chlorine for fluorine, was not effective. Blocking of SH-groups by these compounds seems not to be responsible for the activation.

In ASW containing L-cysteine, FDNB did not activate the eggs. It did not activate also the eggs pretreated with iodoacetamide. These results suggest that FDNB-effect is exerted by releasing F^- in consequence of the reaction with intracellular SH-groups.

Whereas Ca^{2+} ionophore, ionomycin, activated the eggs pretreated with TMB-8 FDNB did not. On the other hand, both the reagents were ineffective to eggs pretreated with cytochalasin B. These results suggest that the step(s) FDNB affects exists before that ionomycin does in the cascade of the cortical reaction.

Acknowledgment

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