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Early transmembrane events in tumour cell responses observed by stopped-flow fluorometry

Naoko Utsunomiya, Masamichi Tsuboi and Mamoru Nakanishi*

Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

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Early transmembrane events of tumour cells (mouse myeloma X5563 and lymphoma RDM4) after binding of a monoclonal antibody against mouse MHC antigen and a mitogenic lectin, Con A, were examined by stopped-flow fluorometry with 3 different fluorescent probes. The results showed that membrane fluidities of the cells increased first after binding of anti H-2K^k monoclonal antibody (11-4.1), then calcium was released from intracellular stores into the cytoplasma, and lastly calcium influx occurred from the external medium into the cytoplasma. While Con A only induced calcium influx from the external medium into the cytoplasma.

Stopped-flow fluorometry *Membrane fluidity* *Calcium influx* *MHC antigen* *Con A* *Tumor cell*

1. INTRODUCTION

There is now considerable evidence from several systems that the rise in cytosolic calcium which follows receptor triggering is preceded by the breakdown of phosphatidylinositol bisphosphate into 1,2-diacylglycerol and inositol triphosphate [1–5]. The latter is known to cause release of calcium from intracellular stores. However, the relationships between such a rise in intracellular free calcium and the early transmembrane events involved in cell activation are still not clear.

To solve such a problem we have used stopped-flow fluorometry for monitoring early transmembrane events involved in cell activation. As the physical changes observed here in tumour cells are very informative, the method can be widely used for studying early transmembrane events in cell activation.

* To whom correspondence should be addressed

Abbreviations: MHC, major histocompatibility complex; Con A, concanavalin A; CTC, chlortetracycline; PBS, phosphate-buffered saline; FCS, fetal calf serum

2. MATERIALS AND METHODS

Monoclonal cell line (11-4.1) was grown in cell cultures [6]. Anti H-2K^k monoclonal antibody (IgG2a) was purified from culture supernatant on a protein A-Sepharose (Pharmacia) column [7]. Myeloma cell X5563 (H-2K^k) was passaged in C3H mice. Lymphoma cell RDM4 (H-2K^k) was grown in cell cultures in RPMI 1640 + 10% FCS. Con A was obtained from EY Lab. Anti H-2D^b monoclonal antibody was obtained from Biotest-Serum-Institute. Quin2AM was purchased from Dojin, A23187 from Calbiochem, RPMI 1640 from Gibco, and CTC from Sigma.

Fluorescence spectra were observed using a Hitachi 650-10S fluorescence spectrophotometer. Stopped-flow fluorescence measurements were taken on a Union Giken RA-401 stopped-flow spectrophotometer in combination with an RA-450 microcomputer system [8]. We used a Hoya Y46 cut-off filter (which allows emitted light with a wavelength longer than 460 nm to enter the detector) in the fluorescence measurements.

Fluorescent compounds were incorporated into X5563 and RDM4 cells by incubation in PBS, with

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10 μM quin2AM for 40 min at 37°C, 50 μM CTC for 30 min at 37°C and 10 μM 2-(1-pyrenebutyryloxy)stearic acid for 40 min at 37°C. Quin2AM was hydrolyzed into quin2 by the cell esterases. These processes were monitored by the shift in the emission spectrum from the peak of quin2AM at 430 nm to the quin2 peak around 490 nm [9,10].

3. RESULTS

3.1. *Calcium influx into target cells*

Fig.1a shows the effect on calcium influx of anti H-2K^k monoclonal antibody in mouse myeloma cells X5563 (H-2K^k) observed by stopped-flow fluorometry. After mixing of anti H-2K^k antibody (6×10^{-8} M) with quin2-loaded X5563 cells (or RDM4 cells) (5×10^6 cells/ml), the fluorescence of the cells rises in 2–3 s, indicating a rise of intracellular free calcium concentration to a new steady-state level (half-life 0.8 s, see fig.1a, upper record). Assuming that all the cells in suspension respond to anti H-2K^k antibody, the total observed fluorescence increase corresponds to a 7–8-fold increase of the intracellular free calcium concentration (initial value 1.0×10^{-7} M) following the procedure of Tsien [9,10]. When EGTA (2 mM) was present in the external medium, the increase in fluorescence almost disappeared within the time range shown in fig.1b. This fact indicates that the fluorescence increase in fig.1a corresponds mainly to the calcium influx from the external medium into the target cells. For a control experiment we also mixed PBS with quin2-loaded X5563 cells, however, no increase in fluorescence was observed as shown in fig.1a (lower record). We further found that the fluorescence intensity of the quin2-loaded cells did not change after mixing with anti H-2D^b monoclonal antibody (6×10^{-8} M) (not shown).

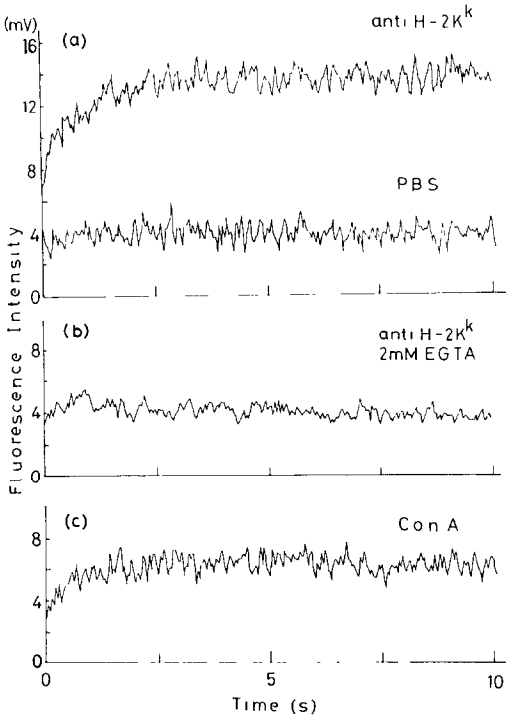

Fig.1. Stopped-flow measurements of the effects of anti H-2K^k antibody and Con A on the fluorescence of quin2 trapped in mouse myeloma X5563 cells. Final concentration: 5×10^6 myeloma cells/ml + 10 μM quin2. Excited at 340 nm. (a) 6×10^{-8} M anti H-2K^k antibody was added to the cells at 25°C. 'PBS' means a control experiment where we mixed PBS alone with X5563 cells containing quin2. (b) The effect of anti H-2K^k antibody on the fluorescence of quin2 where EGTA (2 mM) was present in the external medium. (c) 5×10^{-7} M Con A was added to the cells at 25°C.

These fluorescence increases in target cells also take place after binding with Con A (5×10^{-7} M). Rates of fluorescence increase were dependent on the concentration of Con A. 5×10^{-7} M Con A had a similar effect on the rate of calcium influx to 6×10^{-8} M anti H-2K^k antibody. However, the increase in fluorescence intensity due to Con A was smaller than that with anti H-2K^k antibody.

3.2. *Redistribution of membrane-bound calcium*

Next we measured the effects on CTC fluorescence of anti H-2K^k antibody in the cells, choosing the same antibody concentration (6×10^{-8} M). After anti H-2K^k antibody binding to X5563 cells, the CTC fluorescence decreases with a half-life of 0.27 s, which is appreciably shorter than the lifetime of quin2 fluorescence increase (half-life 0.8 s) (see fig.2). Such a CTC fluorescence intensity is expected to decrease as calcium is released from internal stores into the cytoplasm [11,12]. The observed rate (0.27 s) of this process indicates that the redistribution of calcium from internal stores is more rapid than the overall rate of the calcium influx into the cytoplasm (0.8 s). In addition, there is an impor-

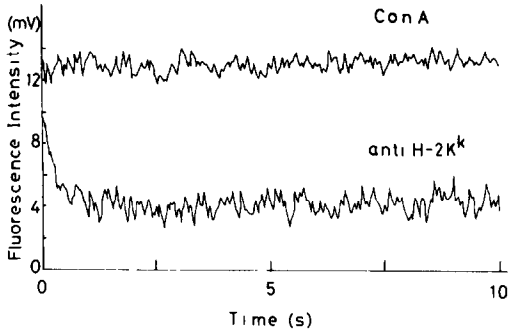

Fig.2. Stopped-flow measurements of effects of anti H-2K^k antibody and Con A on the fluorescence of CTC trapped in myeloma cells. Final concentration: 5×10^6 myeloma cells/ml in which CTC content was 50 μM. 6×10^{-8} M anti H-2K^k antibody or 5×10^{-7} M Con A was added to the cells at 25°C. Excited at 400 nm. CTC fluorescence decreased gradually after mixing cells with protein solution because of the dilution effect. Therefore, recordings were made after subtracting a control curve (mixed with PBS) from each curve by an on-line computer.

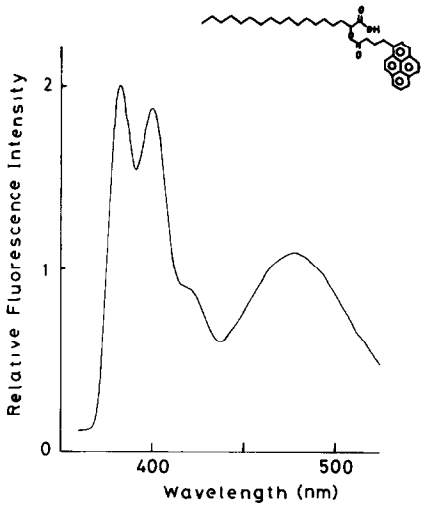

Fig.3. Fluorescence spectrum of 2-(1-pyrenebutyryl-oxy)stearic acid embedded in mouse myeloma X5563 cells at 25°C. Excited at 340 nm.

tant difference between the binding of anti H-2K^k antibody and that of Con A. Con A did not have any effect on CTC fluorescence in X5563 cells as shown in fig.2 (upper curve).

3.3. *Change of membrane fluidity*

Lastly, we attempted to examine a time-dependent change of membrane fluidity in target cell membranes after anti H-2K^k antibody (or Con A) binding. Fluidity change of the tumour cell membranes caused by anti H-2K^k binding can be measured by the amount of 2-(1-pyrenebutyryl-oxy)stearic acid excimer formed in the membranes. Fig.3 shows the fluorescence spectrum of 2-(1-pyrenebutyryloxy)stearic acid embedded in X5563 cell membranes. Two peaks at 382 and 400 nm are assigned to the monomer fluorescence of pyrene and a broad peak around 480 nm to the excimer fluorescence [13,14]. After binding of anti H-2K^k antibody, the excimer fluorescence in the cell membranes rises at 3.4 s^{-1} (lifetime 0.2 s) as shown in fig.4, indicating a rise of membrane fluidity [13,14]. This rate is somewhat faster than that of the release rate of calcium from internal stores into the cytoplasm (CTC fluorescence). Here, Con A has no significant effect on pyrene fluorescence in the cell membranes as shown in fig.4 (lower curve).

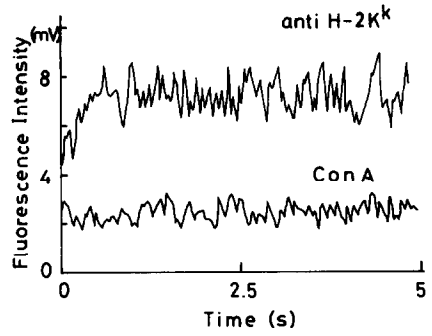

Fig.4. Responses of pyrene excimer fluorescence to anti H-2K^k antibody and Con A. Final concentration: 5×10^6 myeloma cells/ml in which 2-(1-pyrenebutyryl-oxy)stearic acid content is 10 μM. 6×10^{-8} M anti H-2K^k antibody or 5×10^{-7} M Con A was added to the cells at 25°C. Excited at 340 nm. The fluorescence light of wavelengths longer than 460 nm was observed.

4. DISCUSSION

The present experimental results show that anti H-2K^k antibody produces an increase in free calcium in the target tumour cells (X5563 and RDM4 cells, H-2K^k) by both calcium release from internal stores and calcium influx from the external medium. It has also been shown that Con A

binding does not cause a release of calcium from internal stores into cytoplasm but does cause a flow from the external medium. Thus, the total amount of the quin2 fluorescence increase (see fig.1a) is greater for anti H-2K^k antibody binding than for Con A binding. The present results also show that the fluidity of the target cell membranes increases first (half-time 0.2 s) after anti H-2K^k antibody binding to the target cells. Therefore, membrane fluidization is required before an increase in the concentration of intracellular free calcium in the cell responses. We have recently found a similar pattern in some other systems, including, for example, rabbit blood platelets after binding with thrombin (Ohga et al., to be published). The early transmembrane events described here may be taken as having a generality among ligand-receptor interactions in many other systems.

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