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Calcium control of actin–myosin-based contraction in Triton-treated murine bladder tumor cells

O. Yamaguchi, C. Irisawa, K. Baba, Y. Yoshimura, T. Yokota, Y. Shiraiwa

Department of Urology, Fukushima Medical College, Fukushima, 960-12 Japan

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Summary. Using mouse bladder tumor cells (MBT-2 cells) and epithelial cells, the present study evaluated the functional characteristics of the actomyosin system in bladder cancer cells. An immunofluorescence study demonstrated the presence of contractile proteins (actin and myosin) in MBT-2 cells as well as bladder epithelial cells. Triton-treated MBT-2 cells and epithelial cells showed a Ca^{2+}-dependent contraction. This was inhibited by *N*-ethylmaleimide-modified myosin subfragment 1 (NEM-S_1), demonstrating that the interaction between actin and myosin is responsible for the contraction of Triton-treated cells. The extent of Ca^{2+}-dependent contraction was much greater in MBT-2 cells than in epithelial cells. These results suggest that MBT-2 cells possess a locomotive apparatus consisting of actin and myosin, and that Ca^{2+} can activate this actomyosin system, leading to the contraction or active locomotory movement of tumor cells.

Key words: Actin – Calcium ion – Contraction – Locomotion – Murine bladder tumor cells – Myosin

Invasion and metastasis are prominent features of malignant tumors. Recently, evidence has been accumulated suggesting that these characteristics of malignant tumors can be attributed to the locomotive activity of tumor cells [3, 6, 9, 12, 13]. Since the presence of contractile proteins (actin and myosin) in non-muscular mammalian cells [5, 8, 16] as well as cancer cells [2] has been demonstrated, the concepts developed in the studies of muscle contraction have been the basis for the understanding of motility in non-muscle cells. The actomyosin system in non-muscle cells is thus assumed to be the force-generating system for cell locomotion. However, as far as tumor cells are concerned, little is known about the dynamic aspects of this actomyosin system, particularly in urogenital malignant tumor cells.

The present study, using Triton-treated mouse bladder tumor cells, investigated whether Ca^{2+} activates the actomyosin system and induces contraction or microscopic locomotion in bladder cancer cells.

Materials and methods

Mouse bladder tumor (MBT-2) was used in this experiment. This tumor is a poorly differentiated transitional cell carcinoma and has retained its histological characteristics during serial transplantation in syngeneic mice [14]. A single-cell suspension of MBT-2 tumor was prepared according to the procedure of Soloway [14]. Epithelial cells of mouse bladder were used for control experiments. Bladder was taken from a C3H mouse and opened in cold Krebs solution. Epithelium was detached from submucosa and minced. After enzymatic dissociation (0.25% trypsin), epithelial cells were collected by differential centrifugation. MBT-2 cells and epithelial cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum. Cells cultured for 5–6 days on collagen-coated plates (Sumitomo Bakelite) were used for the contraction experiment.

Since the cell membrane is known to form the major obstacle to influences on the actomyosin system from the external medium, the permeabilized cell model obtained from a detergent treatment was used [1, 4, 10]. To prepare permeabilized cells, cells were washed once with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) and treated with an ice-cold solution consisting of 0.02% Triton X-100, 100 mM KCl, 2 mM EGTA, 1 mM dithiothreitol (DTT) and 10 mM imidazole-HCl (pH 7.2) for 5 min. In some cases the concentration of Triton was changed from the standard 0.02% to 0.1% or 0.2% to estimate the effect of Triton concentration on the contractility of cells. After Triton treatment the cells were washed with an ice-cold solution containing 100 mM KCl, 2 mM EGTA and 10 mM imidazole-HCl (pH 7.2) to remove the detergent.

In the contraction experiment, Triton-treated cells were preincubated in Ca^{2+}-free relaxing solution consisting of 1 mM EGTA, 100 mM KCl, 3 mM $MgCl_2$, 3 mM ATP and 10 mM imidazole-HCl at room temperature. Then, the medium was replaced with the activating solution containing Ca^{2+}, and the changes in cell shape continuously recorded using a phase-contrast microscope-video imaging system (NAC). The activating solution contained 100 mM KCl, 10 mM imidazole-HCl, 3 mM $MgCl_2$, 3 mM ATP, 1 mM EGTA and Ca^{2+}. The various Ca^{2+} concentrations in the activating solution

Correspondence to: O. Yamaguchi; FAX 81(245)48-3836

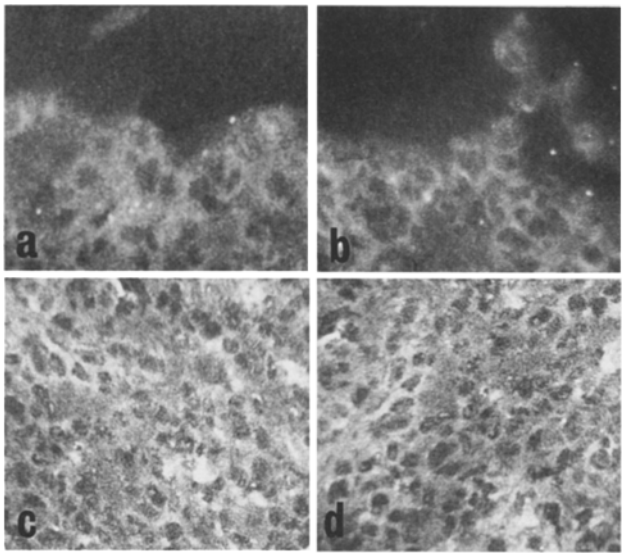

Fig. 1 a–d. Immunofluorescent staining of frozen sections. Actin (**a**) and myosin (**b**) in mouse bladder epithelium. Actin (**c**) and myosin (**d**) in MBT-2 tumor. (Original magnification ×200)

were made by adding $CaCl_2$ to the relaxing solution containing 1 mM EGTA and calculated with an EGTA–Ca EGTA buffer system.

Cells which had been separated from each other were selected for the contraction experiment because preliminary observations showed that within a dense cell population the contraction of individual cells was difficult to measure. The extent of contraction was estimated by measuring the cell area before and after replacing the relaxing solution with the activating solution. In order to measure the cell area, a still picture was printed out from the video recording. The area of the cell was then determined by weighing a cut-out picture of each cell outline.

As a specific inhibitor for the actin–myosin interaction, *N*-ethylmaleimide-modified myosin subfragment 1 (NEM-S_1) was used, prepared by the method of Cande [1]. Myosin subfragment 1 (Sigma) at 5 mg/ml was incubated in 10 mM imidazole-HCl, 0.2 mM DTT with 1 mM freshly dissolved NEM, pH 7.0, at room temperature for 75 min. The reaction was stopped by adding DTT (10 mM), and unreacted NEM was removed by exhaustive dialysis.

An indirect immunofluorescence study was performed to confirm the presence of contractile proteins in both MBT-2 cells and bladder epithelial cells. Anti-actin (Sigma) and anti-myosin (Sigma) which had been developed in rabbit against actin isolated from chicken back muscle and myosin isolated from bovine uterine smooth muscle, respectively, were used as primary antibodies. Frozen mouse bladder wall and MBT-2 tumor were cut in a cryostat at 4–6 μm thickness. The cryostat sections were mounted on glass slides and treated with acetone at 4 °C for 5 min after air drying. They were subsequently washed with PBS. An appropriate dilution (1:40 for anti-actin, 1:10 for anti-myosin) of the rabbit antibody was applied to the sections. After incubation in a moisture chamber at 20 °C for 1 h, the sections were washed with PBS and incubated for 1 h with a 1:80 dilution of goat anti-rabbit globulins (Sigma) coupled

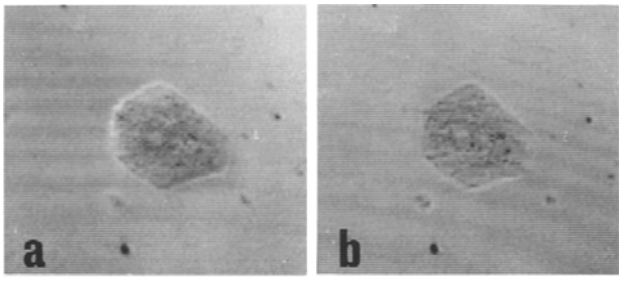

Fig. 2 a, b. Phase-contrast microscopy of Triton-treated epithelial cell. **a** In Ca^{2+}-free relaxing solution; **b** 20 min after replacement with the activating solution containing 5 μM Ca^{2+}. (×200)

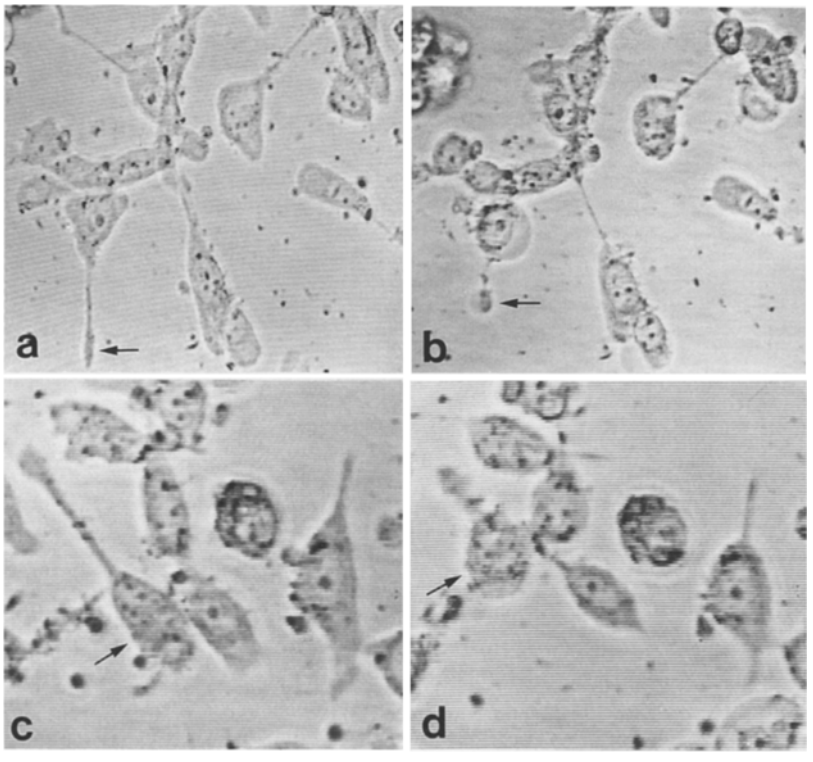

Fig. 3 a–d. Contraction and movement of Triton-treated MBT-2 cells. **a, b** MBT-2 cells before and 15 min after addition of 5 μM Ca^{2+}. *Arrows* indicate the shortening of a cytoplasmic process. **c, d** Before and 15 min after adding 5 μM Ca^{2+}. *Arrows* show the movement of the cell. Other cells exhibit contraction in response to Ca^{2+}. (×400)

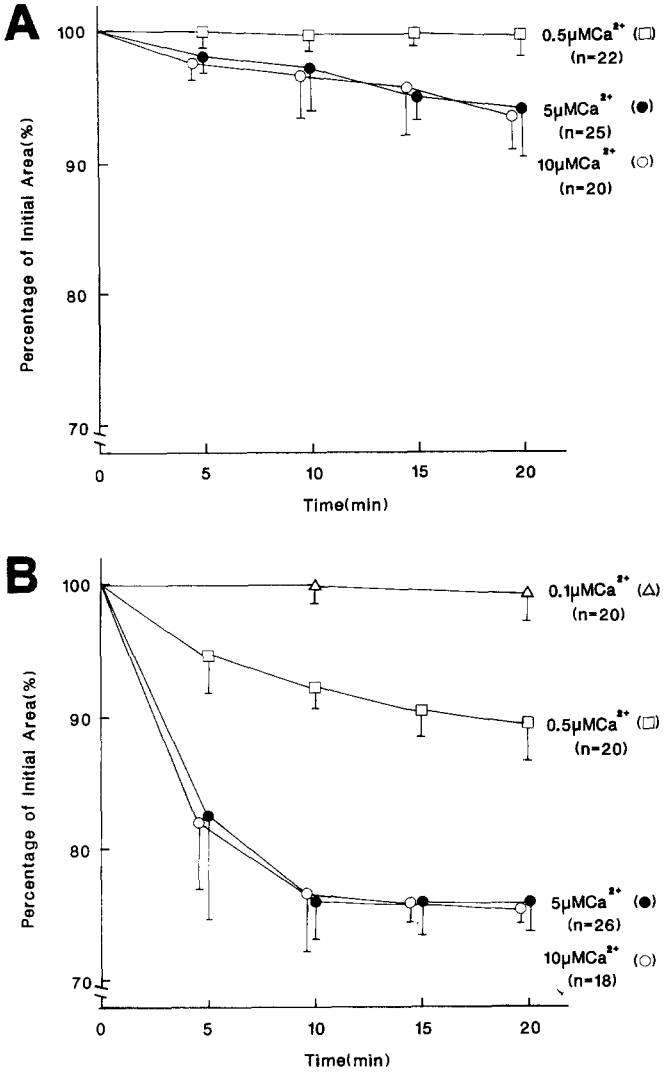

Fig. 4A, B. Dependence of contraction on Ca^{2+} concentration. The response of epithelial cells and MBT-2 cells to Ca^{2+}, respectively

to fluorescein (FITC). The sections were finally washed with PBS and covered with a drop of 80% glycerol in PBS and a coverslip. These samples were examined in an Olympus MMST fluorescence microscope.

Results

Epithelium of mouse bladder as well as MBT tumor showed immunofluorescent staining for actin and myosin (Fig. 1). The staining of contractile proteins in both epithelial cells and MBT-2 cells was strong around the nucleus (which itself was not stained) and particularly at the periphery of the cell. In the sections incubated with rabbit antiserum obtained before immunization, no fluorescence was present.

Triton-treated epithelial cells showed an extremely small contraction when the bathing medium was changed to the activating solution containing Ca^{2+} (Fig. 2). In contrast to epithelial cells, Triton-treated MBT-2 cells

Table 1. Effects of Triton concentration on Ca^{2+}-induced contraction

Triton X-100 concentration (%)	Decrease relative to initial area (%)	
	Epithelial cells	MBT-2 cells
0.02	6.2 ± 3.6 ($n=25$)	24.4 ± 2.1 ($n=26$)
0.1	5.3 ± 2.8 ($n=10$)	25.1 ± 1.6 ($n=14$)
0.2	5.9 ± 3.4 ($n=15$)	24.8 ± 2.5 ($n=10$)

The decrease (%) in cell area at 20 min after addition of 5 μM Ca^{2+} is shown as the mean ± SD. The differences in cell contraction when the Triton concentration was increased to 0.1% and 0.2% were not significant in either group

Table 2. Effect of NEM-S_1 on Ca^{2+}-induced contraction

	Area of cell (μm²)	
	MBT-2 cell ($n=21$)	Epithelial cell ($n=20$)
Control	760.2 ± 164.8	1682.6 ± 455.9
At 20 min after addition of 5 μM Ca^{2+}	767.6 ± 166.8	1688.5 ± 456.9

Values are shown as the mean ± SD

exhibited a marked contraction in response to Ca^{2+} (Fig. 3). This contractile response was characterized by a shrinkage of the cytoplasm. In addition, processes extruding from the cytoplasm sometimes showed a shortening (Fig. 3a, b) In a few cases, as a process shortened, the cell moved toward the end of the process which was attached to the substratum (Fig. 3c, d).

Figure 4 shows the dependence of the contractile response on Ca^{2+} concentration. In Triton-treated epithelial cells (Fig. 4a), 0.5 μM Ca^{2+} did not cause contraction. At 5 of 10 μM Ca^{2+}, the epithelial cells slowly contracted, the decrease in cell area being only 6.2% of the initial area at 20 min after the addition of Ca^{2+} (Fig. 4a). When the concentration of Triton was increased to 0.1% or 0.2%, Triton-treated epithelial cells showed a contraction in response to 5 μM Ca^{2+} identical to that at a Triton concentration of 0.02% (Table 1).

Triton-treated MBT-2 cells (Fig. 4b) began to shrink at a Ca^{2+} concentration of 0.1 μM. At Ca^{2+} concentrations less than 0.1 μM the contraction did not occur. The extent and rate of contraction were increased with an increase in Ca^{2+} concentration. MBT-2 cells contracted maximally within 10 min at 5 μM or more Ca^{2+}. The maximal response estimated by the changes in cell area was approximately a 24% decrease relative to the initial area (Fig. 4b). This contractility of Triton-treated MBT-2 cells was not altered by increasing the Triton concentration up to and including 0.2% (Table 1).

When Triton-treated MBT-2 cells and epithelial cells were preincubated with 1 mg/ml NEM-S_1 for 5 min, the

contraction of cells induced by 5 μM Ca^{2+} was completely inhibited (Table 2). There was no significant difference between the cell areas before and after adding 5 μM Ca^{2+} in either cell group (paired *t*-test).

Discussion

The immunofluorescence study demonstrates that the contractile proteins actin and myosin are present in both MBT-2 cells and bladder epithelial cells. In order to investigate the functional characteristics of this actomyosin system we used the permeabilized cell model obtained by Triton extraction. Recently, Masuda et al. [10] showed that actin and myosin were retained in various non-muscle cells after Triton treatment.

It was also demonstrated in the present study that Triton-treated MBT-2 cells as well as epithelial cells are capable of contraction in the presence of Ca^{2+}, the extent of contraction being dependent on the Ca^{2+} concentration. Since this Ca^{2+}-dependent contraction was inhibited by NEM-S_1, the biochemical interaction between actin and myosin might be responsible for the contraction of Triton-treated cells.

The significant difference between the Triton-treated epithelial cells and MBT-2 cells is that the Ca^{2+}-induced contraction of epithelial cells is much smaller and slower than that of MBT-2 cells. If the cell membrane of epithelial cells is more resistant to Triton treatment, the difference in contractility may appear because both types of cells were treated with the same concentration (0.02%) of Triton. Thus, we briefly examined the effect of Triton concentration on contractility. The results, however, showed that in neither cell group was the extent of Ca^{2+}-induced contraction altered by increasing the Triton concentration. Therefore, the difference in contractility demonstrated here seems to reflect a difference in Ca^{2+} sensitivity between the two cell species. It would be assumed from these results that the actomyosin system in MBT-2 cells is more sensitive to Ca^{2+} than that in bladder epithelial cells.

These dynamic characteristics of the actomyosin system in MBT-2 cells are similar to those in muscle cells, in that Ca^{2+} primarily regulates the function of the contractile apparatus which is mainly composed of actin and myosin [11]. In this respect the actomyosin system in MBT-2 cells is considered as a locomotive apparatus that generates a driving force in the interior of the cell.

With regard to a morphological unit of locomotion, it has been generally accepted that in the cytoplasm of most eukaryotic cells actin is organized into microfilaments which are decorated with myosin and other accessory proteins [7, 8, 16]. The same Ca^{2+}-dependent contraction was also demonstrated in Triton-treated 3T3 fibroblasts [4, 10], which are known to possess actin–myosin-containing microfilaments [8, 16]. Thus, microfilaments seem to be the main morphologically discernible element of this locomotive apparatus.

If human bladder cancer cells and other malignant tumor cells have the same locomotive apparatus as demonstrated in MBT-2 cells, Ca^{2+} can induce locomotory movement, which may result in tumor cell invasion or metastasis. Evidence to support this hypothesis may be drawn from a recent study [15] showing that in cultured human carcinoma and melanoma cells, histamine (a ubiquitous hormone) mobilized intracellular Ca^{2+} and subsequently increased cell migration.

Although this study suggests the functional basis for understanding the locomotory activity of malignant tumor cells in culture, the results can not be directly transferred to the in vivo situation. Further investigation is required to clarify the role of tumor cell motility based on this mechanism in invasion or metastasis.

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Editorial comments

There are still some concerns that should be considered by the authors. For one, many investigators do not agree that a shape change such as observed here should be called a contraction. The time course is far too slow for a conventional contractile response. More importantly it is not clear whether all of the tumor cells responded, i.e. the authors do not indicate whether the analysis was performed on a random field of cells or whether the authors measured shape changes only in "responsive" cells. A statement addressing this point should be included in the final manuscript. Secondly it would be useful if the investigators compared the responses of the tumor cells to smooth muscle cells from the bladder. This cell type more closely resembles the tumor cells morphologically and may represent the cell type from which the tumor arose initially. In addition, comparison of shape changes in tumor cells versus smooth muscle cells might provide some insight as to role of the actomyosin system in "invasiveness". Thirdly the authors should be careful as to the sources of antibody for immunofluorescence; i.e. it is not clear why they employed an antibody to *skeletal* muscle actin and *smooth* muscle myosin. This may explain why such high titers (1:10 dilution) of antibody were required for the immunofluorescence studies. Indeed the authors should make sure that all immunofluorescence is abolished after the serum is absorbed with actin and myosin.

C. R. Scheid, PhD

Reply to the editorial comment

It is clear from the present study that Triton-treated tumor cells showed a marked shrinkage after adding Ca^{2+}. This response may be called contraction. Compared to the conventional contractile response such as muscle cell contraction, the difference in calmodulin or myosin light chain kinase content may explain this slow response of tumor cells.

All Triton-treated tumor cells showed contraction in response to Ca^{2+} (0.5–10 μM). To analyze the contractility of individual cells, the magnification of the microscope was increased. Prior to adding Ca^{2+}, several cells in a given microscopic field were randomly selected for analysis. These target cells were continuously monitored by the television camera during the experiment. The number of target cells obtained from one culture disc was three to five because of the high magnification. Repeating the contraction experiment on other culture discs, approximately 20 cells selected at random were statistically analyzed by a paired *t*-test for the cell area before and after adding Ca^{2+}.

We agree with the comment that the responses of the tumor cells are worth comparing with those of smooth muscle cells from bladder. Such a study would provide more information on the characteristics of the actomyosin system in cancer cells.

As indicate, it is appropriate to use the same source of antibody for the immunofluorescence study. Smooth muscle actin and myosin might be better in this case. However, an antibody to skeletal muscle actin is also suitable for this study because the molecular structure of actin is approximately the same in a wide variety of cell species. In addition, the working dilution of anti-myosin (1:10) was determined by indirect immunofluorescent labeling of cultured fibroblasts.

O. Yamaguchi, MD