All-or-none augmentation of Ca^{2+} sensitivity in α -toxin-permeabilized single smooth muscle cells from guinea-pig taenia caecum

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1 Isolated smooth muscle cells from guinea-pig taenia caecum were permeabilized by use of *Staphylococcus aureus* α -toxin, and the sarcoplasmic reticulum Ca^{2+} store was depleted by exposure to $0.1\ \mu\text{M}$ A23187.

2 Shortening of α -toxin-permeabilized single smooth muscle cells was induced by increasing free Ca^{2+} but was not induced by $0.2\ \mu\text{M}$ free Ca^{2+} .

3 Shortening of the permeabilized cells was caused by application of acetylcholine (ACh) with free Ca^{2+} concentration held at $0.2\ \mu\text{M}$. Permeabilized smooth muscle cells responded to $0.3\ \mu\text{M}$ or $1\ \mu\text{M}$ ACh with $0.2\ \mu\text{M}$ Ca^{2+} with maximal shortening. The concentration-response relationship to ACh had a very steep slope and the cell shortening appeared to be an all-or-none response rather than a graded response, as was the shortening of intact cells to ACh.

4 The shortening of permeabilized cells was also induced by application of guanosine 5'-triphosphate (GTP) with $0.2\ \mu\text{M}$ free Ca^{2+} , showing an all-or-none response. The threshold concentration of GTP that induced an all-or-none response was between $10\ \mu\text{M}$ and $30\ \mu\text{M}$.

5 These results suggest that Ca^{2+} sensitivity is augmented by stimulation of the muscarinic receptor or GTP-binding protein(s) in an all-or-none manner. It seems probable that this contributes to the all-or-none response to ACh in intact smooth muscle cells.

Keywords: Ca^{2+} sensitivity; smooth muscle cells; all-or-none response; permeabilization; α -toxin; acetylcholine; muscarinic receptor; GTP-binding protein; taenia caecum

Introduction

We reported previously that isolated single smooth muscle cells from guinea-pig taenia caecum showed an all-or-none response to acetylcholine (ACh) (Mita & Uchida, 1987; 1988; 1991; Mita *et al.*, 1988; 1993). These are the first smooth muscle cells to demonstrate a large and prompt all-or-none response repeatedly and with the same dose-response relationship. Moreover, we found that isolated single smooth muscle cells from a different organ, the fundus of guinea-pig stomach, also showed an all-or-none response to ACh (Mita *et al.*, 1993).

Ca^{2+} plays a central role in the contraction of smooth muscle induced by neurotransmitters and hormones. The rise in cytoplasmic free Ca^{2+} triggers smooth muscle contraction by activating myosin light chain kinase. However, the relationship between cytosolic Ca^{2+} concentration and the contraction level is not always constant (Bradley & Morgan, 1987; Karaki *et al.*, 1988; Karaki, 1989), suggesting the existence of additional mechanisms through which agonists can modulate the sensitivity of the contractile apparatus to Ca^{2+} . Augmentation of Ca^{2+} sensitivity in receptor-operated contraction has been studied in α -toxin-permeabilized whole smooth muscles (Nishimura *et al.*, 1988; Kitazawa *et al.*, 1989; 1991). This mechanism was shown to be mediated by GTP-binding protein(s) on the basis of the observation that GTP or $\text{GTP}\gamma\text{S}$ lowers the Ca^{2+} concentration necessary for the contraction. We demonstrated previously that the shortening of α -toxin-permeabilized single smooth muscle cells from two different organs, the fundus of the guinea-pig stomach and the guinea-pig taenia caecum, was induced by increasing the concentration of free Ca^{2+} and the cells responded to either $0.3\ \mu\text{M}$ or $0.6\ \mu\text{M}$ Ca^{2+} with maximal shortening, showing an all-or-none response, but did not respond to $0.2\ \mu\text{M}$ Ca^{2+} or

lower (Ono *et al.*, 1992; Oishi *et al.*, 1992; Mita *et al.*, 1994). This result suggested that the threshold concentration of Ca^{2+} for developing cell shortening lies between $0.3\ \mu\text{M}$ and $0.6\ \mu\text{M}$ Ca^{2+} , and α -toxin-permeabilized cells show an all-or-none response as a result of a slight increase in the intracellular free Ca^{2+} level over the threshold concentration. Moreover, the addition of ACh ($10\ \mu\text{M}$) or GTP ($100\ \mu\text{M}$) resulted in a decrease in the concentration of Ca^{2+} required to trigger a threshold response from $0.6\ \mu\text{M}$ to $0.2\ \mu\text{M}$ and from $0.3\ \mu\text{M}$ to $0.1\ \mu\text{M}$, but the concentration of Ca^{2+} required to trigger the maximal shortening was not changed (Ono *et al.*, 1992; Oishi *et al.*, 1992; Mita *et al.*, 1994). Therefore, the cell shortening showed a graded response. These results suggested that these permeabilized cells retained their receptor function, and that augmentation of Ca^{2+} sensitivity was induced by the stimulation of muscarinic receptors with ACh and GTP-binding proteins with GTP. In this paper, in order to elucidate further the mechanisms determining the threshold for the shortening of intact single smooth muscle cells in response to ACh, we studied the relationship between an all-or-none response to ACh and the augmentation of Ca^{2+} sensitivity using the shortening of α -toxin-permeabilized single smooth muscle cells from guinea-pig taenia caecum.

Methods

Cell isolation

Isolated smooth muscle cells from guinea-pig taenia caecum were prepared by the method described previously (Mita & Uchida, 1987). Strips of taenia caecum were minced and incubated with 0.1% collagenase (about $250\ \text{u ml}^{-1}$) in Ca^{2+} -free solution containing 0.2% trypsin inhibitor and 1.0% bovine serum albumin at 37°C for 80 min with gentle stirring.

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The suspension of cells was centrifuged at 120 g and the precipitate was suspended in Ca^{2+} -free solution. The cells were dispersed by pipetting the suspension several times with a wide-bore pipette. The medium was HEPES-Tyrod solution composed of (mM): NaCl 137, KCl 2.7, CaCl_2 1.8, MgCl_2 1.0, glucose 5.6 and HEPES 4.2, pH 7.4 at 37°C (normal solution). The Ca^{2+} -free solution had the same composition except that CaCl_2 was omitted.

Measurement of the shortening of single cells

Shortening of individual isolated smooth muscle cells was measured by the method of Mita & Uchida (1987). The cell suspension was placed on a silicon-coated glass slide with a cover slip, and the cells were perfused continuously with Ca^{2+} -free solution at 30°C. The perfusion fluid was introduced from one side and was blotted off by filter paper from the other side of the slide. The silicon-coated glass slide was placed on a temperature-controlled stage (30°C) of a phase contrast microscope. The shortening of individual cells observed by phase contrast microscopy was recorded on video tape, and the cell length was measured with an ARUGUS-10 Image Analyzer (Hamamatsu Photonics K.K., Japan).

Cell permeabilization

In order to ensure that intact isolated cells did not respond to extracellular Ca^{2+} , individual cells were perfused before permeabilization with the cytosolic substitution solution containing an ascending concentration of free Ca^{2+} buffered at 10 nM to 30 μM . The cytosolic substitution solution was composed of (mM): potassium propionate 114, MgCl_2 4, ATP 4, creatine phosphate 10, EGTA 1, and piperazine- $\text{N,N}'$ -bis(2-ethanesulphonic acid) (PIPES) 20, (pH 7.1 at 30°C). Free Ca^{2+} concentrations were determined according to the method of Harafuji & Ogawa (1980). The free Ca^{2+} concentration of cytosolic substitution solution with no added Ca^{2+} (Ca^{2+} -free) was estimated to be 2.5 nM. After washing the cells with the Ca^{2+} -free cytosolic substitution solution, the cells were permeabilized by perfusing with the solution containing α -toxin, 10 $\mu\text{g ml}^{-1}$, and Ca^{2+} , 0.1 μM , for 5 min. After washing the cells with Ca^{2+} -free cytosolic substitution solution for 3 min, permeabilized cells were perfused continuously with Ca^{2+} -free cytosolic substitution solution containing 0.1 μM of the Ca^{2+} -translocator A23187 for 10 min. The permeabilized cells were then perfused with cytosolic substitution solution containing Ca^{2+} , 0.2 μM , and A23187 for 5 min to eliminate the function of sarcoplasmic reticulum Ca^{2+} stores. Itoh *et al.* (1985) previously reported that 0.1 μM A23187 had no effect on the Ca^{2+} -tension relation in saponin-treated skinned muscle tissues of the rabbit mesenteric artery and did not change the resting tension, but inhibited caffeine-induced contractions, suggesting that these effects of A23187 (0.1 μM) may be due to an ability to act selectively on the Ca^{2+} store site and increase the Ca^{2+} leakage from the store. Therefore, with A23187 (0.1 μM) treatment the Ca^{2+} stores will be depleted. The first shortening was evoked by perfusion with cytosolic substitution solution containing increasing concentrations of ACh or GTP with 0.2 μM Ca^{2+} and A23187. After washing with Ca^{2+} -free cytosolic substitution solution plus A23187 for 15 min and cytosolic substitution solution containing 0.2 μM Ca^{2+} and A23187 for 5 min, the second cell shortening in response to ACh or GTP in the presence of 0.2 μM Ca^{2+} and A23187 was determined. After washing with Ca^{2+} -free cytosolic substitution solution plus A23187 for 20 min, the effectiveness of permeabilization was determined by exposing the cells to the cytosolic substitution solution containing free Ca^{2+} buffered at 1 μM .

Chemicals

Staphylococcus aureus α -toxin (Ono *et al.*, 1992) was kindly provided by Drs M.K. Uchida and K. Oishi (Department of

Molecular Pharmacology, Meiji College of Pharmacy). Collagenase (Type I), soybean trypsin inhibitor (Type II-S), creatine phosphate and adenosine 5'-triphosphate dipotassium (ATP) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Guanosine 5'-triphosphate disodium salt (GTP) was from Boehringer Mannheim. Bovine serum albumin (Fraction V) and potassium propionate were from Nakarai Tesque (Kyoto, Japan). Acetylcholine (Ovisot) was from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). N-2-hydroxyethylpiperazine- N' -2-ethanesulphonic acid (HEPES), piperazine- $\text{N,N}'$ -bis(2-ethanesulphonic acid) (PIPES), O,O'-bis(2-aminoethyl)ethyleneglycol- N,N,N',N' -tetraacetic acid (EGTA) and inositol 1,4,5-trisphosphate (IP_3) were from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of reagent grade.

Results

Figure 1 shows the effect of A23187 on inositol 1,4,5-trisphosphate (IP_3)-induced shortening of α -toxin-permeabilized smooth muscle cells. The decrease in the length of the cells induced by 10 μM IP_3 with free Ca^{2+} concentration held at 0.2 μM was $15.5 \pm 1.5\%$ ($n=3$). However, the shortening of permeabilized cells was not induced by 0.2 μM Ca^{2+} alone (Mita *et al.*, 1994). The cell shortening in response to IP_3 with

Figure 1 Effect of A23187 on inositol 1,4,5-trisphosphate (IP_3)-induced shortening of α -toxin-permeabilized single smooth muscle cells. Permeabilized cells were perfused with cytosolic substitution solution containing 0.2 μM free Ca^{2+} for 5 min. None of the cells responded to 0.2 μM Ca^{2+} . The first cell shortening was then evoked by perfusion with 10 μM IP_3 in the presence of 0.2 μM Ca^{2+} . After washing with Ca^{2+} -free cytosolic substitution solution for 10 min, permeabilized cells were treated with Ca^{2+} -free cytosolic substitution solution containing 0.1 μM A23187 for 10 min and were perfused with cytosolic substitution solution containing Ca^{2+} (0.2 μM) in the presence of A23187 (0.1 μM) for 5 min. The cells were then exposed to 10 μM IP_3 in the presence of Ca^{2+} (0.2 μM) and A23187. After washing with cytosolic substitution solution containing Ca^{2+} (0.2 μM) and A23187 for 5 min, cells were further exposed to 30 mM caffeine in the presence of Ca^{2+} (0.2 μM) and A23187. Values are means $\pm$ s.e. ($n=3$). The ordinate scale represents the decrease in cell length as a percentage of original length. After exposure to A23187, shortening in response to IP_3 or caffeine was abolished.

$0.2 \mu\text{M}$ Ca^{2+} was completely inhibited by $0.1 \mu\text{M}$ A23187. Moreover, permeabilized cells did not show caffeine (30 mM) induced cell shortening on treatment with A23187. Therefore, to maintain cytoplasmic Ca^{2+} constant in permeabilized cells, subsequent experiments were carried out in cytosolic substitution solutions containing A23187 ($0.1 \mu\text{M}$), which depleted the sarcoplasmic reticulum of Ca^{2+} .

The shortening of permeabilized cells was evoked by increasing the concentration of ACh, when the concentration of free Ca^{2+} was held at $0.2 \mu\text{M}$ Ca^{2+} in the presence of A23187. Permeabilized smooth muscle cells responded to $0.3 \mu\text{M}$ or $1 \mu\text{M}$ ACh in an all-or-none manner (Figure 2a). These threshold concentrations of ACh were identical with those in intact cells showing an all-or-none response (Mita & Uchida, 1987; 1988; Mita *et al.*, 1988). Permeabilized cells were almost completely relaxed again by washing with the Ca^{2+} -free cytosolic substitution solution. Moreover, the cells responded repeatedly to the same threshold concentration of ACh in the same manner (Figure 2). Thus, although individual permeabilized cells showed an all-or-none response to ACh with $0.2 \mu\text{M}$ Ca^{2+} , the average contractile response of all the cells was graded because of the difference in the threshold concentration of ACh (Figure 2b). The maximal decreases in the length of the cells induced by ACh with $0.2 \mu\text{M}$ Ca^{2+} in the first and second cell shortening were $17.3 \pm 0.1\%$ and $15.8 \pm 1.4\%$, respectively. The difference between these values was not significant. The shortening of α -toxin-permeabilized single cells was also induced by application of GTP with $0.2 \mu\text{M}$ free Ca^{2+} in the presence of A23187, showing an all-or-none response (Figure 3a). The threshold concentration of GTP that induced an all-or-none response was either $10 \mu\text{M}$ or $30 \mu\text{M}$. However, the average contractile response of all the cells was graded because of the difference in the threshold concentration of GTP (Figure 3b). The maximal decrease in the length of the cells induced by GTP with $0.2 \mu\text{M}$ Ca^{2+} was $17.3 \pm 3.1\%$. This value was not significantly different from that induced by ACh.

Figure 2 Contractile response to acetylcholine (ACh) of α -toxin-permeabilized single smooth muscle cells in cytosolic substitution solution with the free Ca^{2+} concentration held at $0.2 \mu\text{M}$ in the presence of A23187. Permeabilized cells were treated with Ca^{2+} -free cytosolic substitution solution containing $0.1 \mu\text{M}$ A23187 for 10 min and were perfused with cytosolic substitution solution containing $0.2 \mu\text{M}$ Ca^{2+} and $0.1 \mu\text{M}$ A23187 for 5 min. The first shortening was evoked by successive perfusion with cytosolic substitution solution containing increasing concentrations of ACh with Ca^{2+} ($0.2 \mu\text{M}$) in the presence of A23187 ($\circ$). After washing with Ca^{2+} -free cytosolic substitution solution in the presence of A23187 for 15 min and perfusing cytosolic substitution solution containing Ca^{2+} ($0.2 \mu\text{M}$) and A23187 for 5 min, the second cell shortening in response to ACh in the presence of Ca^{2+} ($0.2 \mu\text{M}$) and A23187 was observed ($\square$). (a) A typical shortening of an α -toxin-permeabilized smooth muscle cell in response to increasing concentrations of ACh. (b) Mean contractile responses of all the cells. Results were obtained with cells from different preparations made on different days. Points and bars are means $\pm$ s.e. ($n=3$). This plot shows that the dose-response relationship in the first and second cell shortening were the same. The ordinate scale represents the maximal shortening of the first dose-response curve to ACh as 100%.

Figure 3 Contractile response to guanosine 5'-triphosphate (GTP) of α -toxin-permeabilized single smooth muscle cells in cytosolic substitution solution with the free Ca^{2+} concentration held at $0.2 \mu\text{M}$ in the presence of A23187. Permeabilized cells were treated with Ca^{2+} -free cytosolic substitution solution containing $0.1 \mu\text{M}$ A23187 for 10 min and were perfused with cytosolic substitution solution containing $0.2 \mu\text{M}$ Ca^{2+} and $0.1 \mu\text{M}$ A23187 for 5 min. Shortening was evoked by successive perfusion with cytosolic substitution solution containing increasing concentrations of GTP with Ca^{2+} ($0.2 \mu\text{M}$) in the presence of A23187 (Δ). (a) A typical shortening of an α -toxin-permeabilized smooth muscle cell in response to increasing concentrations of GTP. (b) Mean contractile responses of all the cells. Results were obtained with cells from different preparations made on different days. Points and bars are means $\pm$ s.e. ($n=4$). The ordinate scale represents the maximal shortening to GTP as 100%.

Discussion

The addition of IP_3 ($10 \mu\text{M}$) with Ca^{2+} ($0.2 \mu\text{M}$) in α -toxin permeabilized smooth muscle cells caused cell shortening (Figure 1). This result suggests that the function of intracellular Ca^{2+} store in permeabilized cells remains intact. In fact, because the pores formed in the membrane by α -toxin are too small to allow an α -toxin molecule itself to pass, α -toxin attaches only to the plasma membrane and does not reach intracellular organelles (Ahnert-Hilger & Gratzl, 1988; Bhakdi & Tranum-Jensen, 1991). However, the cell shortening in response to IP_3 with Ca^{2+} ($0.2 \mu\text{M}$) was abolished by treatment with A23187 ($0.1 \mu\text{M}$). Furthermore, permeabilized smooth muscle cells also did not respond to caffeine (30 mM) on treatment with A23187. Kitazawa *et al.* (1989) showed that the sarcoplasmic reticulum was depleted of Ca^{2+} by A23187 in α -toxin-permeabilized guinea-pig portal vein and A23187 abolished IP_3 -induced Ca^{2+} release. It is well known that two mechanisms of Ca^{2+} release from the Ca^{2+} store exist in smooth muscle. They are Ca^{2+} -induced Ca^{2+} release (CICR), which is sensitive to caffeine, and IP_3 -induced Ca^{2+} release (IICR). It has been shown that the Ca^{2+} store in taenia caecum consists of two compartments, one with both CICR and IICR mechanisms, and the other with only an IICR mechanism (Iino, 1987; Yamazawa *et al.*, 1992). Our results suggest that neither mechanism of Ca^{2+} release function on treatment with A23187 in α -toxin-permeabilized smooth muscle cells, and cytoplasmic Ca^{2+} is maintained constant under these conditions. Therefore, we can determine the real Ca^{2+} sensitization stimulated by ACh or GTP without Ca^{2+} mobilization.

α -Toxin-permeabilized single smooth muscle cells showed repeatedly an all-or-none response to $0.3 \mu\text{M}$ or $1 \mu\text{M}$ ACh in the presence of Ca^{2+} ($0.2 \mu\text{M}$) (Figure 2). A similar all-or-none response was also induced by GTP in the presence of Ca^{2+} ($0.2 \mu\text{M}$) (Figure 3). These results suggest that the Ca^{2+} sensitivity is augmented in an all-or-none manner by stimulation of muscarinic receptors or GTP-binding proteins under a constant Ca^{2+} concentration. This is the first observation that Ca^{2+} sensitization of smooth muscle contraction is induced by the stimulation of receptors in an all-or-none manner. The augmentation of Ca^{2+} sensitivity stimulated by an α -adrenoceptor agonist, phenylephrine, in the contraction of α -toxin-permeabilized whole tissues (guinea-pig portal vein) was in-

duced concentration-dependently in the presence of $0.5 \mu\text{M}$ free Ca^{2+} (Kitazawa *et al.*, 1991). The difference in these results may be explained by the distinction between whole tissues and isolated cells used in experiments. In general, intact whole muscle tissues including taenia caecum respond concentration-dependently to ACh. On the other hand, as we found previously, isolated intact individual smooth muscle cells from guinea-pig taenia caecum and the fundus of the stomach respond to ACh in an all-or-none manner. However, in these cells the average response of all the cells is graded because of the difference in the threshold concentration of ACh (Mita & Uchida, 1987; Mita *et al.*, 1988; 1993).

The threshold concentrations of ACh causing an all-or-none response in the intact cells were identical with those showing all-or-none augmentation of Ca^{2+} sensitivity in α -toxin-permeabilized cells. This result suggests that the all-or-none response of intact cells to ACh may be connected with the all-or-none augmentation of Ca^{2+} sensitivity to ACh. However, the shortening was greater to ACh in these permeabilized cells when Ca^{2+} concentration was raised beyond $0.2 \mu\text{M}$ (Mita *et al.*, 1994). It is suggested that the Ca^{2+} sensitivity is augmented to ACh in an all-or-none manner, but the magnitude of the maximal shortening induced by ACh depends on the Ca^{2+} concentration. The maximal decrease in the length of the permeabilized cells induced by increasing the concentration of free Ca^{2+} in the absence and presence of $10 \mu\text{M}$ ACh was $38.6 \pm 2.9\%$ and $44.0 \pm 2.4\%$, respectively; the difference between these values was significant at $P=0.01$ on a paired basis ($n=6$; Mita *et al.*, 1994). On the other hand, the maximal shortening of the all-or-none response to ACh in intact single cells was $54.9 \pm 1.0\%$ ($n=85$; Mita & Uchida, 1988). The intact cells shorten significantly more than the permeabilized cells in response to muscarinic receptor activation. Furthermore, the shortening of the permeabilized cells in response to Ca^{2+} only reached a maximum after 3–4 min, but the intact cells shortened within 30 s. It seems that the difference in these rates of shortening may result from different rates of Ca^{2+} mobilization. Ca^{2+} release from the sarcoplasmic reticulum, and influx from the extracellular space may raise $[\text{Ca}^{2+}]_i$ more rapidly in the normal cells than Ca^{2+} diffusion into the permeabilized cells (Mita & Uchida, 1988). Intact cells may also have a positive feedback mechanism of Ca^{2+} entry, as shown in ileal smooth muscle by Inoue & Isenberg (1990), who reported that the inward current through muscarinic receptor-activated non-selective cation channels was enhanced by a rise in intracellular Ca^{2+} , thus maintaining Ca^{2+} influx by sustaining depolarization. In sum, it seems that the intact cells may shorten more, and more rapidly than the permeabilized cells due to the rapid Ca^{2+} mobilization with these mechanisms when muscarinic receptor stimulation augments the Ca^{2+} sensitivity.

We do not think that all the cells which constitute the whole tissue show an all-or-none Ca^{2+} sensitization to ACh. Only about 50% of the intact cells responded to ACh (Mita & Uchida, 1987). This phenomenon may be due to the pattern of innervation of smooth muscle cells. Burnstock & Iwayama (1971) reported that there are three types of cells in smooth muscle: directly innervated cells (key cell), coupled cells and indirectly coupled cells. Therefore, only the key cell may contain the receptors and signal transduction pathways to respond to ACh. The all-or-none response to individual cells may be masked by the tissue behaving as a functional syncytium with

the cells being affected by their neighbours. Moreover, we cannot observe an all-or-none Ca^{2+} sensitization in intact cells, because muscarinic receptor activation involves a rapid, large increase of intracellular Ca^{2+} concentration above threshold through Ca^{2+} release from sarcoplasmic reticulum and Ca^{2+} influx from the extracellular space. Only the key cell, when isolated alone and permeabilized, shows the augmentation of Ca^{2+} sensitivity to ACh in an all-or-none manner.

At present, we cannot explain the molecular mechanisms of development of Ca^{2+} sensitization in an all-or-none manner. However, the addition of GTP also induced an all-or-none augmentation of Ca^{2+} sensitivity (Figure 3). This result suggests that the augmentation of Ca^{2+} sensitivity is evoked as a result of activation of GTP-binding proteins, and that an all-or-none Ca^{2+} sensitization to ACh is induced downstream from the GTP-binding proteins. Others have suggested that Ca^{2+} sensitization results from imbalance between the activities of myosin light chain kinase and myosin light chain phosphatase (Stull *et al.*, 1990), which may involve inhibition of myosin phosphatase via arachidonic acid (Somlyo *et al.*, 1989; Kitazawa *et al.*, 1991; Kubota *et al.*, 1992; Gong *et al.*, 1992), a GTP-binding protein coupled mechanism possibly involving small GTP-binding proteins such as rhoA p21 (Hirata *et al.*, 1992) and ras p21 (Satoh *et al.*, 1993), or phosphorylation of myosin light chain kinase by Ca^{2+} /calmodulin-dependent protein kinase II (Tang *et al.*, 1992; Tansey *et al.*, 1992). Nishimura *et al.* (1988) reported that protein kinase C is involved in the increase of Ca^{2+} sensitivity in α -toxin-permeabilized smooth muscle tissues. However, protein kinase C is not involved in the muscarinic receptor-operated augmentation of Ca^{2+} sensitivity in permeabilized single smooth muscle cells (Oishi *et al.*, 1992). A regulatory mechanism that is independent of myosin light chain phosphorylation has also been reported (Oishi *et al.*, 1991) and this mechanism may possibly be involved in the Ca^{2+} sensitization. In addition, it has been suggested that thin filament-associated proteins such as caldesmon and calponin may regulate smooth muscle contraction on the side of the actin filament (Winder & Walsh, 1993). Further study is clearly needed to identify fully the mechanisms of development of Ca^{2+} sensitization to ACh in α -toxin-permeabilized single smooth muscle cells.

In conclusion, it is suggested that intact smooth muscle cells show an all-or-none shortening to ACh as a result of a slight increase in the intracellular Ca^{2+} level over the threshold concentration and an all-or-none augmentation of Ca^{2+} sensitivity via the activation of GTP-binding protein(s) when muscarinic receptor stimulation reaches the threshold to evoke Ca^{2+} influx and IP_3 -induced Ca^{2+} release. It should be pointed out, however, that the single cells are shortening under unloaded conditions, and the affect of load has not been assessed.

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