Br. J. Pharmacol. (1992), **106**, 539–543

Receptor-coupled shortening of α-toxin-permeabilized single smooth muscle cells from the guinea-pig stomach

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1 Isolated single smooth muscle cells from the fundus of the guinea-pig stomach were permeabilized by use of *Staphylococcus aureus* α-toxin. Receptor-coupled shortening of individual cells was monitored under phase contrast microscopy.

2 Most of the isolated cells responded to 0.6 μM Ca^{2+}, but not to 0.3 μM Ca^{2+}, with a resulting maximal shortening to approximately 65% of the resting cell length. The contractile activity of these permeabilized cells lasted for several hours and repeated shortening was readily achieved after washing out.

3 Addition of acetylcholine (ACh) at a maximal concentration (10 μM) resulted in a marked decrease in the concentration of Ca^{2+} required to trigger a threshold response from 0.6 μM to 0.2 μM, and 1 mM guanosine 5′-diphosphate (GDP) blocked this decrease. Moreover, treatment with 100 μM guanosine 5′-triphosphate (GTP) mimicked the action of ACh.

4 Addition of 100 μM inositol 1,4,5-trisphosphate ($InsP_3$) with 0.2 μM Ca^{2+} did not cause cell shortening, whereas 10 μM ACh with 0.2 μM Ca^{2+} did, suggesting that $InsP_3$-induced Ca^{2+} release is not involved in ACh-operated cell shortening.

5 The present study demonstrates an α-toxin-permeabilized single smooth muscle cell preparation which retains its receptor function and also provides an insight into mechanisms leading to augmentation of Ca^{2+} sensitivity by stimulation of muscarinic receptors or GTP-binding proteins.

Keywords: Ca^{2+} sensitivity; permeabilization; smooth muscle cells; muscarinic receptor; GTP-binding protein

Introduction

One approach to elucidate the intracellular mechanisms underlying smooth muscle contraction involves permeabilization of the plasma membrane, which permits manipulation of the intracellular environment. α-Toxin, a cytolytic exotoxin secreted by *Staphylococcus aureus* that forms pores of 2–3 nm diameter in the plasma membrane, has been used as a tool for selectively permeabilizing the plasma membrane of, for example, secretory cells to small molecules with molecular weights up to a thousand daltons (Füssle *et al.*, 1981; Ahnert-Hilger *et al.*, 1985; Hohman, 1988). This technique has been applied to whole smooth muscles and preparations where intact receptors and signal transduction systems were retained (Nishimura *et al.*, 1988; Kitazawa *et al.*, 1989; 1991). It thus provides a valuable new means of investigating the mechanisms of stimulus-contraction coupling. One of the main shortcomings when applying this technique to intact smooth muscle preparations is the difficulty of ensuring homogeneous permeabilization of all the cells. In order to overcome this problem, we have extended the method to isolated single smooth muscle cells. We have shown for the first time that α-toxin-permeabilized single muscle cells retain their receptor function. Moreover, these permeabilized single cells from the fundus of the stomach are suitable for studying the increase in Ca^{2+}-sensitivity induced by stimulation of muscarinic receptors or guanosine 5′-triphosphate (GTP)-binding proteins.

Methods

Preparation of α-toxin

α-Toxin was purified from the culture supernatant of *Staphylococcus aureus* strain Wood 46 by the method of Hohman (1988). The haemolytic activity of α-toxin was checked with rabbit erythrocytes. Purified α-toxin was 80–85% pure as judged by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The purified toxin permeabilized rat basophilic leukaemia (RBL-2H3) cells to [^{3}H]-adenine but did not permit release of lactate dehydrogenase. Protein was determined according to Lowry *et al.* (1951) with bovine serum albumin used as standard.

Cell isolation

Smooth muscle cells were isolated from the fundus of the guinea-pig stomach by a modification of the method of Mita & Uchida (1987). Guinea-pigs (400–800 g) were killed by a blow on the neck and exsanguinated. The stomach was removed immediately and the fundus was resected. The serosa and mucosa were dissected from the muscle layer of the fundus. Strips of fundic muscle were suspended in normal N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES)-Tyrode solution bubbled with air at 37°C for 90 min and then transferred to Ca^{2+}-free HEPES-Tyrode solution (Ca^{2+}-free solution) bubbled with air at 37°C for 75 min. The normal solution had the following composition (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). The Ca^{2+}-free solution had the same composition as normal HEPES-Tyrode solution except that $CaCl_2$ was omitted. The tissue was then minced and incubated with 0.5 mg ml^{-1} collagenase (127.5 unit ml^{-1}), 0.125 mg ml^{-1} elastase and 0.1 mg ml^{-1} trypsin inhibitor in 2 ml of Ca^{2+}-free solution containing 4% bovine serum albumin at 37°C for 80 min with gentle stirring. The suspension was diluted with 8 ml of Ca^{2+}-free solution containing 4% bovine serum albumin and was centrifuged at 120 *g*. This washing procedure was repeated once more. The cells were suspended in 4 ml of Ca^{2+}-free solution and dispersed with a wide-bore pipette. The viability of isolated single cells,

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measured by trypan blue exclusion, was more than 90%. The mean length of intact single cells obtained by this method was 112 ± 3 μm (mean ± s.e.mean, $n = 21$). Approximately 50% of the intact smooth muscle cells retained the ability to contract in response to acetylcholine (ACh); these cells were spindle shaped and bright, and their surface was smooth.

Measurement of shortening of single cells

Shortening of individual isolated smooth muscle cells was measured by the method of Mita & Uchida (1987). In brief, the cell suspension was placed on a silicon-coated glass slide with a cover slip. The cells were perfused continuously with Ca^{2+}-free solution at 30°C. The perfusion fluid was introduced from one side of the slide and blotted off with filter paper from the other side. The silicon-coated glass slide was placed on the temperature-controlled stage (30°C) of a phase contrast microscope. The shortening of individual cells observed by phase contrast microscopy was recorded on a video tape and the cell length was measured with the 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 had the following composition (mM): potassium propionate 114, glycoletherdiamine-N,N,N',N'-tetraacetic acid (EGTA) 1, $MgCl_2$ 4, ATP 4, creatine phosphate 10, and piperazine-*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). After washing the cells with the Ca^{2+}-free cytosolic substitution solution (the free Ca^{2+} concentration was estimated to be 2.5 nM), permeabilization was achieved by perfusing with the solution containing 10 μg ml^{-1} α-toxin and 0.1 μM Ca^{2+} for 3 min. α-Toxin was then washed out by perfusing with the Ca^{2+}-free cytosolic substitution solution, and the effectiveness of permeabilization was checked by exposing the cells to the cytosolic substitution solution containing an ascending concentration of free Ca^{2+} buffered at 10 nM to 3 μM, and observing shortening. After extensive washing with the Ca^{2+}-free cytosolic substitution solution for 20 min, responding cells were isolated and used for experimentation. Since the contractile response occurred within 90 s in all cases, the time of exposure at each concentration of Ca^{2+} was 120 s. The first shortening was evoked by perfusion with the cytosolic substitution solution containing an ascending concentration of free Ca^{2+} buffered at 10 nM to 3 μM. After extensive washing with the Ca^{2+}-free cytosolic substitution solution for 20 min, a second shortening was evoked with an ascending concentration of Ca^{2+} in the presence or absence of ACh, GTP, or guanosine 5'-diphosphate (GDP).

Drugs used

Collagenase (type 1), bovine serum albumin (fraction V), creatine phosphate and adenosine 5'-triphosphate dipotassium salt (ATP) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); elastase, guanosine 5'-diphosphate dilithium salt (GDP), and guanosine 5'-triphosphate disodium salt (GTP) were from Boehringer; acetylcholine (ACh, Ovisot) was from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan); inositol 1,4,5-trisphosphate ($InsP_3$) was from Wako Pure Chemical Industries (Osaka, Japan); N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), piperazine-N,N'-*bis* (2-ethanesulphonic acid) (PIPES), and glycoletherdiamine-N,N,N',N'-tetraacetic acid (EGTA) were from Dojindo Laboratories (Kumamoto, Japan); potassium propionate was from Nakarai Tesque (Kyoto, Japan). All other chemicals were of reagent grade.

Results

Figure 1 shows a typical shortening of an α-toxin-permeabilized isolated smooth muscle cell from the fundus of the guinea-pig stomach induced by increasing free Ca^{2+} in the cytosolic substitution solution. In most cases, 0.6 μM Ca^{2+} caused maximal shortening although 0.3 μM or lower Ca^{2+} did not. In all experiments, the contractile response occurred within 90 s and reached a steady state within 60 s. The cells were relaxed again by washing with the Ca^{2+}-free cytosolic substitution solution. In a control study in which the same procedure was carried out without α-toxin, Ca^{2+} in concentrations up to 30 μM did not cause cell shortening. The contractile activity was abolished by removal of ATP and creatine phosphate from the cytosolic substitution solution.

Shortening could be repeatedly evoked by the same threshold concentration of Ca^{2+} for a period of 3 h, but incomplete relaxation in the presence of the Ca^{2+}-free cytosolic substitution solution resulted in a reduction in the absolute magnitude of subsequent responses (Figure 2A). The data shown in Figure 2A were, therefore, normalized by taking the baseline and the maximal shortening of each cell as 0 and 100%, respectively (Figure 2B). Threshold responses to Ca^{2+} were reduced to 0.2 μM from 0.6 μM in the presence of ACh (10 μM) (Figures 2 and 3). The normalized concentration-response relationships to Ca^{2+} in the presence of ACh were identical in repeated experiments despite incomplete relaxation between cycles (Mita & Uchida, 1987).

Addition of 1 mM GDP to the perfusion solution blocked the sensitization to Ca^{2+} induced by ACh (10 μM) (Figure 3). In order to determine if this effect was evoked as a result of

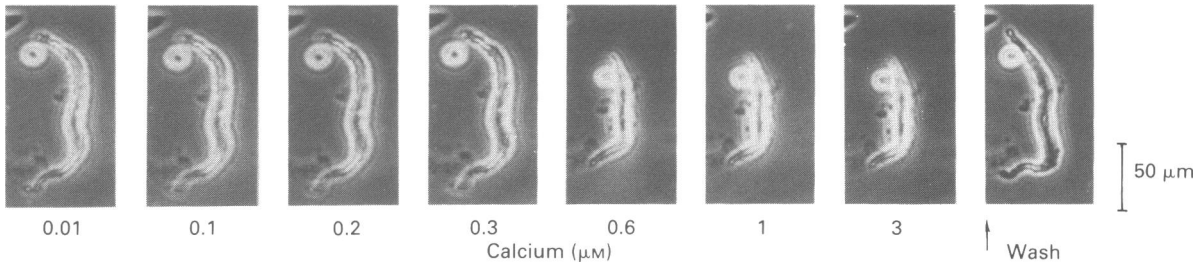

Figure 1 Phase contrast photomicrographs showing the typical shortening of an α-toxin-permeabilized single smooth muscle cell from the fundus of guinea-pig stomach in response to increasing free Ca^{2+}. After permeabilization and extensive washing with the Ca^{2+}-free cytosolic substitution solution for 20 min, cell shortening was evoked by perfusion with the cytosolic substitution solution containing an ascending concentration of free Ca^{2+} buffered at 10 nM to 3 μM. The cell was re-extended after washing with the Ca^{2+}-free cytosolic substitution solution for 20 min. The bar represents 50 μm.

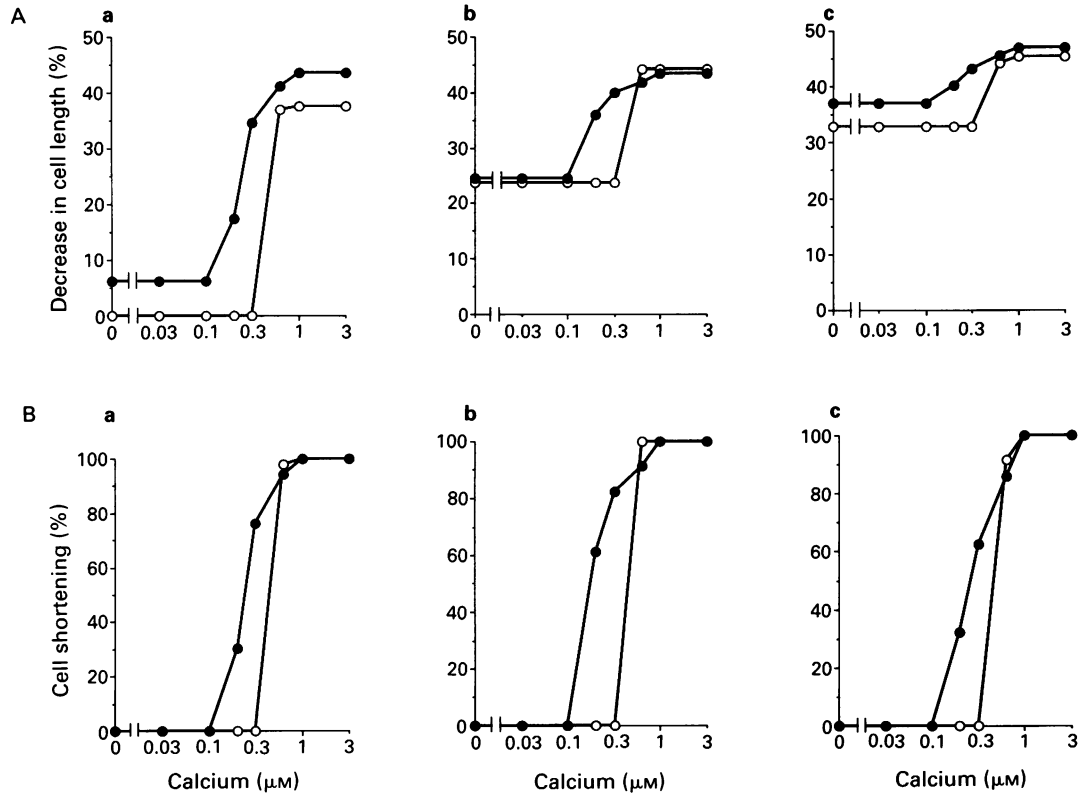

Figure 2 Responses of an α-toxin-permeabilized smooth muscle cell to repeated addition of free Ca^{2+} in the presence (●) and absence (○) of acetylcholine (ACh, 10 μM). (A) After washing the α-toxin-permeabilized smooth muscle cell with the Ca^{2+}-free cytosolic substitution solution for 20 min, the cell was perfused with the cytosolic substitution solution containing an ascending concentration of free Ca^{2+} buffered at 10 nM to 3 μM. The cell was allowed to re-extend by washing with the Ca^{2+}-free cytosolic substitution solution for 20 min, and then perfused with the cytosolic substitution solution containing an ascending concentration of free Ca^{2+} in the presence of ACh (a). This procedure was repeated twice more (b, 2nd; c, 3rd). The ordinate scale in A represents the changes in cell length as a percentage of the original cell length. (B) The data shown in (A) normalized by taking the baseline and the maximal shortening as 0 and 100%, respectively, in each case.

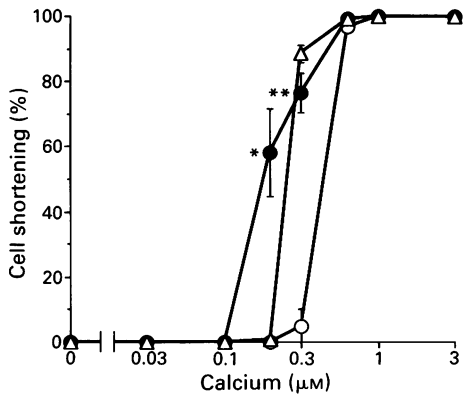

Figure 3 Effect of acetylcholine (ACh) on Ca^{2+}-induced shortening of α-toxin-permeabilized single smooth muscle cells and its blockade by guanosine 5'-diphosphate (GDP). After washing the α-toxin-permeabilized smooth muscle cell with the Ca^{2+}-free cytosolic substitution solution for 20 min, the first shortening was evoked by perfusion with the cytosolic substitution solution containing an ascending concentration of free Ca^{2+} buffered at 10 nM to 3 μM (○). The cells were allowed to re-extend by washing with the Ca^{2+}-free cytosolic substitution solution for 20 min, and a second shortening was evoked as before but in the presence of 10 μM ACh (●). After washing for 20 min, the next shortening was evoked in the presence of 10 μM ACh plus 1 mM GDP (△). The values were plotted by taking the baseline and the maximal shortening of each cell as 0 and 100%, respectively. Points and vertical bars represent means ± s.e.means from five independent experiments. Statistical significance was determined by Student's *t* test (paired): $*P<0.02$; $**P<0.01$.

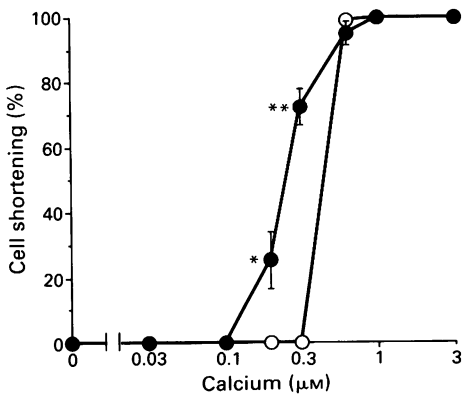

Figure 4 Effect of guanosine 5'-triphosphate (GTP) on Ca^{2+}-induced shortening of α-toxin-permeabilized single smooth muscle cells. After washing the α-toxin-permeabilized smooth muscle cell with the Ca^{2+}-free cytosolic substitution solution for 20 min, the first shortening was evoked by perfusion with the cytosolic substitution solution containing an ascending concentration of free Ca^{2+} buffered at 10 nM to 3 μM (○). The cells were allowed to re-extend by washing with the Ca^{2+}-free cytosolic substitution solution for 20 min, and the next shortening was evoked by an ascending concentration of Ca^{2+} in the presence of 100 μM GTP (●). GTP alone without Ca^{2+} did not influence cell length. The values were plotted by taking the baseline and the maximal shortening of each cell as 0 and 100%, respectively. Points and vertical bars represent means ± s.e.means from six independent experiments. Statistical significance was determined by Student's *t* test (paired): $*P<0.05$; $**P<0.001$.

activation of a GTP-binding protein(s), the effect of GTP was investigated. As shown in Figure 4, 100 μM GTP decreased the threshold concentration for Ca^{2+} from 0.6 μM to 0.2 μM.

Discussion

To date, there have been no reports of isolated single smooth muscle cells being permeabilized with α-toxin. The present study indicates for the first time that α-toxin is a useful tool to permeabilize isolated smooth muscle cells and still retain receptor and signal transduction systems. Detergents such as saponin and Triton X-100 have been widely used for the permeabilization of intact smooth muscle strips and isolated smooth muscle cells (Endo *et al.*, 1977; Gordon, 1978; Obara & Yamada, 1984). A major disadvantage of these chemically skinned muscles is a loss of receptor function (Itoh *et al.*, 1983; Somlyo *et al.*, 1985). Furthermore, detergents destroy the membranes of intracellular organelles (Ahnert-Hilger *et al.*, 1985; Knight & Scrutton, 1986) and interfere with proteins involved in stimulus-contraction coupling.

α-Toxin is a cytolytic exotoxin secreted by *Staphylococcus aureus* that selectively permeabilizes the plasma membranes of eucaryotic cells to small molecules such as ions and nucleotides (Füssle *et al.*, 1981; Ahnert-Hilger *et al.*, 1985; Hohman, 1988). The concentration of ions and small molecules in the cell cytosol can therefore be controlled precisely and specific molecules can be introduced directly.

Addition of 0.6 μM free Ca^{2+} resulted in cell shortening and the removal of Ca^{2+} resulted in relaxation, directly supporting the general idea that contractile activity is determined by increasing the level of intracellular free Ca^{2+} (Bolton, 1979). Our results reported here are in agreement with the calcium sensitivity (half-maximal contraction in the range from 0.3 to 1 μM) reported by others using α-toxin-permeabilized smooth muscle tissues (Nishimura *et al.*, 1988; Kitazawa *et al.*, 1989; 1991). However, one of the obvious discrepancies is that the concentration-response relationship to free Ca^{2+} was very steep in the present study, showing an all-or-none rather than a graded response (Mita & Uchida, 1991).

Augmentation of Ca^{2+}-sensitivity in receptor-operated contraction has been documented in studies using intact smooth muscles (Karaki *et al.*, 1988) and intact α-toxin-permeabilized smooth muscles (Nishimura *et al.*, 1988; Kitazawa *et al.*, 1989), and this was shown to be mediated by GTP-binding protein(s). In our present study, muscarinic receptor stimulation by ACh decreased the concentration of Ca^{2+} required for the threshold response and consequently, converted it to a graded rather than an all-or-none response. Furthermore, addition of GTP mimicked the ability of ACh to enhance sensitivity to Ca^{2+}. These findings suggest that a GTP-binding protein(s) regulates the sensitivity of the contractile proteins to Ca^{2+}. Nonhydrolyzable GTP analogues such as GTPγS are commonly used to activate GTP-binding proteins because GTP is promptly hydrolyzed by endogenous GTPase activity. However, in the present system, GTP itself was sufficient for activation, probably because it was supplied continuously in the perfusate. The increase in sensitivity to Ca^{2+} reported here has also been observed in α-toxin-permeabilized intact smooth muscles (Nishimura *et al.*, 1988; Kitazawa *et al.*, 1989; 1991).

The heterotrimeric signal transducing GTP-binding proteins are now well known to couple cell surface receptors to target enzymes such as phospholipase C and adenylate cyclase (Verghese *et al.*, 1987; Freissmuth *et al.*, 1989). It has been shown that stimulation of various cell surface receptors leads to an activation of phospholipase C via a GTP-binding protein (Cockcroft & Gomperts, 1985; Ashkenazi *et al.*, 1989). The degradation of phosphatidylinositol 4,5-bisphosphate by phospholipase C leads to the production of two second messengers $InsP_3$ and diacylglycerol. $InsP_3$ releases Ca^{2+} from intracellular Ca^{2+} storage sites in various types of smooth muscle (Suematsu *et al.*, 1984; Bitar *et al.*, 1986), whereas diacylglycerol activates protein kinase C in a concerted manner with membrane phospholipids and Ca^{2+}. We found that addition of 100 μM $InsP_3$ in the presence of 0.2 μM free Ca^{2+} did not cause cell shortening (data not shown) although 10 μM ACh in the presence of 0.2 μM free Ca^{2+} did (Figure 2). These results suggest that $InsP_3$-induced Ca^{2+} release is not involved in the receptor-coupled shortening of the permeabilized cells. Our observation is therefore different from those obtained with rabbit permeabilized pulmonary and mesenteric arteries and pig portal vein, where $InsP_3$ in the range 0.5–30 μM caused tension development (Somlyo *et al.*, 1985; Itoh *et al.*, 1985; Kitazawa *et al.*, 1989). The phasic contractions of these smooth muscles were reported to be dependent on Ca^{2+} release from intracellular Ca^{2+} stores (Itoh *et al.*, 1985; Iino *et al.*, 1988). Therefore, there is some dispute as to the contribution of intracellular stored Ca^{2+} to contraction. However, at present, we cannot eliminate an alternative possibility that the lack of response to $InsP_3$ is simply due to the inability to produce a significant rise in the Ca^{2+} level because of the diffusion of Ca^{2+} out of permeabilized cells.

In conclusion, our present study using α-toxin-permeabilized single smooth muscle cells provides direct evidence that receptor-operated augmentation of Ca^{2+} sensitivity is a cellular event. This preparation may prove valuable in investigating further the mechanisms underlying receptor-operated augmentation of Ca^{2+} sensitivity.

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(Received January 2, 1992
Revised February 10, 1992
Accepted February 28, 1992)