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HSP27 IS A MEDIATOR OF SUSTAINED SMOOTH MUSCLE CONTRACTION IN RESPONSE TO BOMBESIN

Khalil N. Bitar [1] *, Mark S. Kaminski [2], Nabil Hailat [3], Kemp B. Cease [2] and John R. Strahler [3]

[1] Division of Pediatric Gastroenterology, University of Michigan Medical Center, Ann Arbor, MI 48109-0658

[3] Division of Pediatric Hematology University of Michigan Medical Center, Ann Arbor, MI 48109

[2] Department of Internal Medicine, Division of Hematology/Oncology, University of Michigan Medical Center, Ann Arbor, MI 48109-0724

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We have identified the low MW 27 kD heat shock protein as a major phosphoprotein constituent of smooth muscle and have investigated its potential role in agonist induced smooth muscle contraction. The neuropeptides bombesin and substance P, which are present in neurons of the anorectal region, induce contraction of isolated smooth muscle cells from this region by activating different intracellular pathways. Substance P-induced contraction is 1,4,5-inositol trisphosphate (IP_3)/calmodulin dependent, while contraction induced by bombesin is mediated by a protein kinase C (PKC) -dependent pathway. The sustained contraction induced by bombesin or exogenous PKC was blocked by preincubation of cells with monoclonal antibodies to hsp27, while the transient contraction induced by substance P or IP_3 was unaffected by the antibodies. Preincubation with isotype matched control antibodies had no inhibitory effect on contraction induced in response to the agents used. These data support a novel role for hsp27 in the non calmodulin mediated sustained contraction induced by bombesin or PKC.

Agonist induced contraction in a number of smooth muscle cells has been associated with a rise in intracellular Ca^{++} concentration [Ca^{++}i] (1, 2, 3, 4, 5). The rise in [Ca^{++}i] activates the calmodulin-dependent enzyme, myosin light chain kinase (6, 7, 8), which results in phosphorylation of 20 kD myosin light chains (mlc). It is known that the interaction of actin and myosin, which results in contraction through a calmodulin dependent process, is regulated by actin ATPase activity induced by phosphorylation of myosin light chain. Numerous proteins have been shown to act as substrates for PKC but the precise pathway leading to muscle contraction in response to PKC mediated agonists is not well understood. We have investigated the hypothesis

* To whom correspondence should be addressed.

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that the sustained contraction induced by bombesin relies upon activation of a set of intracellular proteins different in part from those involved in substance P induced contraction. The predominant phosphorylated polypeptides at basal state consisted of a group of three 27 kD polypeptides determined to be the low molecular weight 27 kD heat shock protein (hsp 27) by immunoblotting with an hsp27 specific antibody (2B4-123). Contraction induced by bombesin and PKC is blocked by preincubation of the cells with the monoclonal antibody to hsp27, while contraction in response to substance P and IP_3 is unaffected by the antibody. These data support a role for hsp27 as an important intermediate in the sustained contraction induced by bombesin and PKC.

Materials and Methods

Isolation of Rabbit rectosigmoid The internal anal sphincter, consisting of the distal most 3 mm of the circular muscle layer, ending at the junction of skin and mucosa, was removed by sharp dissection and a 5 cm length of the rectosigmoid orad to the junction was dissected and digested to yield isolated smooth muscle cells. Cells were isolated as previously described (9). The tissue was incubated for two successive 60-min periods at 31°C in 15 ml of HEPES buffer containing 0.1% collagenase (150 U/mg, Worthington CLS Type II) and 0.01% soybean trypsin inhibitor. At the end of the second enzymatic incubation period, the medium was filtered through 500-μm Nitex. The partially digested tissue left on the filter was washed four times with 50 ml of collagenase-free buffer solution. Tissue was then transferred into 15 ml of fresh buffer solution and incubated for 30 min to allow the cells to disperse spontaneously. Only the cells that dissociated spontaneously in enzyme-free solution were used for functional studies. Cells were counted in a hemocytometer and diluted as needed. Each rectosigmoid yielded 5-10 x 10^6 cells.

Preparation of permeable smooth muscle cells

To test substances such as Ins(1,4,5)P_3 , PKC or the monoclonal antibody, which do not readily pass across the cell membrane, smooth muscle cells were made permeable without affecting their overall function (10). The partly digested muscle tissue was washed with 50 ml of a cytosol-like enzyme-free medium with the following composition : NaCl(20mM), KCl(100mM), $MgSO_4$(5.0mM), NaH_2PO_4(0.96mM), $NaHCO_3$(25.0mM), EGTA (1.0)mM, $CaCl_2$ (0.48mM). The medium contained 2% bovine serum albumin and is equilibrated with 5% O_2/95% CO_2 to maintain a pH of 7.2. Muscle cells were allowed to disperse spontaneously in this medium and were harvested by filtration on 500um Nitex mesh. Isolated cells were permeabilized by incubation for 3 min in saponin (75μg/ml). The cell suspension was then washed in cytosol-like buffer. The cell suspension was filtered through 10-μm Nitex mesh; cells retained on the filter were washed free of saponin and resuspended in the cytosolic buffer containing antimycin (10 μM), ATP (1.5 mM), and an ATP-regenerating system consisting of creatinine phosphate (5 mM) and creatinine phosphokinase (10 units/ml).

Measurement of Contraction. Muscle cells were usually examined within 30 min of dispersion. Aliquots consisting of 2.5 x 10^4 cells in 0.5 ml of medium were added to 0.1 ml of a solution containing agonists, or combinations of agonists and antagonists. In kinetic experiments the reaction was interrupted at various intervals (5 sec to 8 min) by the addition of 0.1 ml of acrolein at a final concentration of 1%. In experiments involving bombesin, substance P or combination of contractile agonists and antagonists the reaction was interrupted at 30 sec. Individual cell length was measured by computerized image micrometry. The average length of cells in the control state or after addition of test agents was obtained from 50 cells encountered randomly in successive microscopic fields. The contractile response was defined as the decrease in the average length of the 50 cells and was expressed as the percent change from control length (10).

Incubation with ^{32}P- orthophosphate and sample preparation. Tissue strips (2mm wide, 25-30 mg wet weight) were incubated in oxygenated HEPES buffered (12mM, pH 7.2), phosphate-free RPMI 1640 (0.5 ml) with ^{32}P- orthophosphate (250 μCi/ml) for 2 h at 37°C under 100% O_2. Tissue was frozen in acetone/dry ice and homogenized with a ground glass pestle in a microfuge tube in 10 volumes of lysis buffer (9 M urea, 2% Nonidet P-40, 2% 2-mercaptoethanol, 2% ampholytes, Pharmacia-LKB pH 3.5-10, and 50 mM NaF). Following centrifugation at 13,000 x g for 3 min, soluble polypeptides were separated by 2-D PAGE using carrier ampholyte

based isoelectric focusing and sodium dodecyl sulfate PAGE in a 11.4-14% acrylamide gel gradient as previously described (11). Gels were dried and ^{32}P- polypeptides visualized by autoradiography on Kodak X-Omat film.

Development of a mouse monoclonal antibody to hsp27 . A mouse monoclonal antibody (2B4-123) was developed using a 15 amino acid synthetic peptide corresponding to residues 123 to 137 of the predicted amino acid sequence for human hsp27. This sequence is within the C-terminal domain of hsp27 which is highly conserved among species. For immunization, the cysteine at residue 15 of the synthetic peptide (residue 137 of hsp27) was conjugated to thyroglobulin. Antisera collected prior to fusion reacted with hsp27 in western blot analysis under both non-denaturing and denaturing electrophoretic conditions. Hybridoma supernatants were initially screened for reactivity with the synthetic peptide by ELISA. Positive clones were screened by western blot analysis of heat shocked K562 cell proteins following separation both by isoelectric focusing under non-denaturing conditions and by denaturing SDS gel electrophoresis, as well as by 2D PAGE. Affinity purified monoclonal anti-hsp27 was obtained from ascites using Protein G Sepharose. The monoclonal antibody is of the IgG1 subclass. Mouse antiserum was obtained following immunization with a 15 amino acid synthetic peptide corresponding to residues 1-15 of human hsp27. The synthetic peptide also contained a C-terminal cysteine for conjugation of the synthetic peptide to thyroglobulin for immunization.

Identification of hsp27-like proteins in 2D gels of rectosigmoid smooth muscle. Tissue strips were labelled with ^{32}P- orthophosphate and proteins (120 ug) were separated by 2-D PAGE. Immunublots were made by electroblotting onto nitrocellulose and probing with a mouse IgG1 monoclonal antibody to a 15 amino acid sequence within the conserved C-terminal domain of human hsp27. Second antibody was goat anti-mouse IgG horseradish peroxidase conjugate. Following staining, the blot was marked with india ink containing trace amounts of radioactivity for registration purposes and an autoradiograph prepared.

Results

Kinetics of contraction of smooth muscle cells. Smooth muscle cells from the rectosigmoid area contracted in response to substance P and bombesin in a concentration dependent manner (data not shown). Substance P-induced contraction peaked and decreased in a time dependent manner, while bombesin-induced contraction peaked and remained elevated for up to 8 minutes (Fig. 1). The time course of contraction that is seen with substance P is similar to that observed with exogenous IP_3. There is a peak response followed by a steady decline for up to 8 minutes. PKC

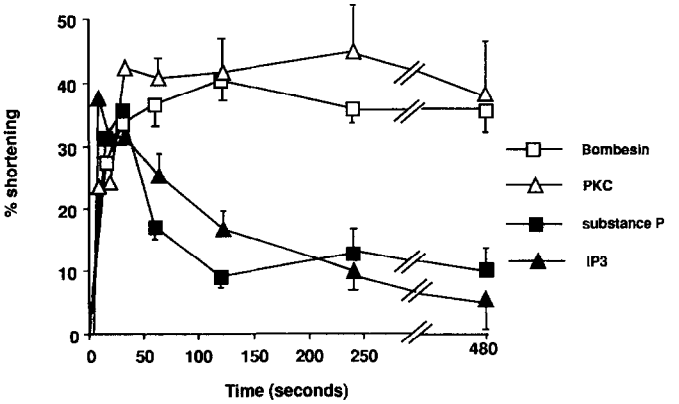

Figure 1. Agonist induced contraction of isolated rectosigmoid smooth muscle cells. Cells were incubated with bombesin and substance P or permeabilized cells were incubated with PKC and IP_3 for the indicated time. Data are expressed as mean ± SEM of 3 experiments. The contractile response is defined as the percent decrease from control length. Bombesin (-□-), 10^{-6} M; substance P (-■-), 10^{-6} M; PKC, (-△-), 100u/mM; IP_3 (-▲-), 10^{-6} M.

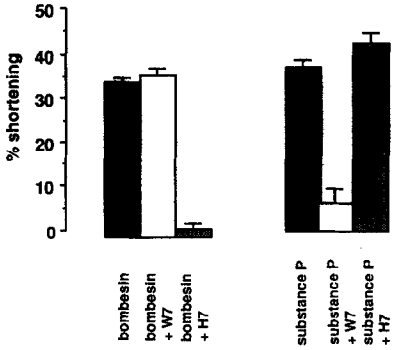

Figure 2. Inhibition of contraction by W7 and H7. Preincubation of the cells for 60 seconds with the calmodulin antagonist W7 (10^{-9}M) blocked maximal substance P-induced contraction, but had no effect on bombesin-induced contraction. Preincubation of the cells for 60 seconds with the PKC antagonist H7 (10^{-6}M) blocked the bombesin-induced contraction and had no effect on the substance P-induced contraction. Data are expressed as mean ±SEM of 3 experiments.

induced contraction remained elevated for up to eight minutes which is similar to the kinetics of contraction induced by bombesin. Bombesin-induced contraction was blocked by the PKC antagonist H7, but was not affected by the calmodulin antagonist W7, whereas substance P-induced contraction was blocked by W7 and not H7 (Fig. 2). The calmodulin antagonist W7 (10^{-9}M) inhibited the maximal contraction induced by IP_3 but not contraction caused by PKC, while the PKC antagonist H7 (10^{-6}M) inhibited the maximal contraction induced by PKC but not contraction caused by IP_3 (data not shown). These differences in the characteristics of contraction induced by bombesin and substance P suggest that they could be mediated by different subsets of proteins.

Identification of Proteins in the Two-dimensional Gel Pattern: The phosphorylation pattern of smooth muscle proteins from the rectosigmoid area of the rabbit was obtained by metabolically labelling tissue with ^{32}P orthophosphate for 2 h. Phosphorylated proteins were analyzed by 2-D PAGE. The predominant phosphorylated polypeptides at basal state consisted of a group of three 27 kD polypeptides (spots b,c,d) (Fig. 3), a 20 kD peptide identified by immunoblotting as myosin light chain (mlc) and a group of 50 kD polypeptides. The 27 kD phosphoproteins are the most predominant after short term (7 min) labeling. Their migration pattern in 2-D gels is similar to a group of polypeptides observed in pre B lymphoblastoid cells which we have identified as the low molecular weight hsp27 (12, 13).

In an effort to determine the identity of spots b, c, and d we noted a similar pattern of 27 kD phosphorylated polypeptides in rat myoblasts (14) and other cell types (15, 16, 17, 18) following heat shock. The identity of spots b, c, d on 2D-PAGE gels was then determined by immunoblotting with an hsp27 specific antibody (2B4-123). Smooth muscle proteins from the rectosigmoid area of the rabbit which were metabolically labelled with ^{32}P orthophosphate were separated by 2-D PAGE and immunublots prepared. The mouse IgG1 monoclonal antibody used was specific for a 15 amino acid sequence from the C-terminal domain of human hsp27 which is highly conserved among species. The antibody detected three 27 kD polypeptides (Fig. 4A, spots a,b,c). Two of the polypeptides (b and c) detected on the immunoblot coincided with

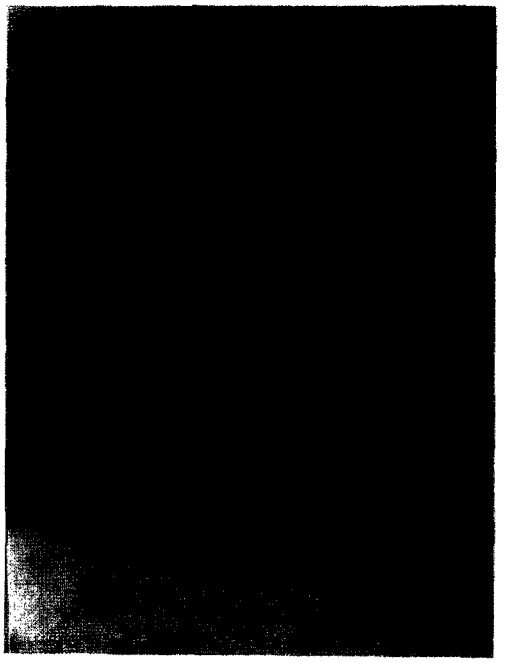

Figure 3. Phosphorylation pattern of smooth muscle proteins in rectosigmoid smooth muscle. The phosphorylation pattern was determined, by metabolic labelling with 32 P-orthophsophate, followed by 2-D PAGE. After metabolic labelling, the predominant phosphorylated polypeptides at basal state consist of a group of three 27 kD polypeptides (spots b,c,d). A 20 kD peptide identified by immunoblotting as myosin light chain (mlc) and a group of 50 kD polypeptides. The 27kD phosphoproteins are the most predominant short term labelling.

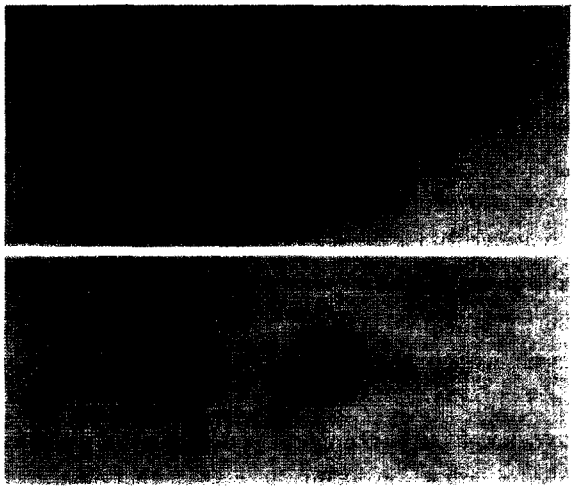

Figure 4. Identification of hsp27-like proteins in rectosigmoid smooth muscle. Tissue strips were labelled with ^{32}P- orthophosphate and proteins (120 µg) were separated by 2-D PAGE as described in methods. An immunublot was made by electroblotting onto nitrocellulose and probing with a mouse IgG1 monoclonal antibody 2B4-123 to human hsp27. A: Close-up section of immunoblot of proteins from rectosigmoid. B: Autoradiograph of the same gel. Large arrows identify hsp27 isoforms. Spot a represents the non-phosphorylated form and spots b, c and d the phosphorylated forms of hsp27 or an hsp27-like protein expressed in rectosigmoid smooth muscle. The d isoform was not visualized on the western blot. Small arrows identify immunoreactive non-phosphorylated proteins detected on the blot which are not seen in human lymphoid cells following stress (Strahler, unpublished).

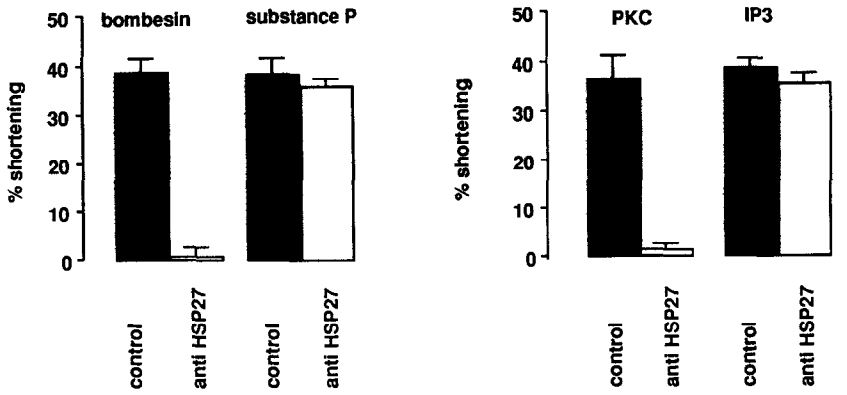

Figure 5. Inhibition of bombesin-induced contraction by mAb 2B4-123 in permeabilized cells. Cells were permeabilized with saponin and incubated with mAb 2B4-123 (1:10,000) or control mAb, MB1, for 1 hour. Bombesin (10^{-6}M) or substance P (10^{-6}M) or IP_3 (10^{-6}M) or PKC (100u/ml) were added as described in the methods section. Data are expressed as mean ±SEM of 3 experiments.

phosphorylated polypeptides b and c of Fig. 4B. The third spot (a) corresponds to non-phosphorylated hsp27. In addition, our hsp27 monoclonal antibody detected a 25 kD and two 26.5 kD nonphosphorylated polypeptides (Fig. 4). Phosphoproteins b and c show a striking similarity in migration pattern to spots 6 and 5 of Rasmussen's groups ((19) Fig. 2,5) which were observed, along with other proteins, to undergo increased phosphorylation during sustained contraction of tracheal smooth muscle in response to carbachol. The pattern of these phosphoproteins on 2D gels and their immunoreactivity strongly supports their identification as forms of hsp27.

Identification of a functional role for hsp27 in smooth muscle contraction: We investigated the possibility that hsp27 might be involved in agonist induced contraction. We have used a functional assay, utilizing permeabilized smooth muscle cells (20), to test the effect of the hsp27 antibodies on contraction induced by either bombesin or substance P. Preincubation of permeablized smooth muscle cells with affinity purified monoclonal antibody to hsp27 (2B4-123) for 60 min had no effect on cell length of isolated permeabilized cells in the absence of agonist (not shown). However, the monoclonal anti-hsp27 antibody inhibited bombesin and PKC-induced contraction while substance P and IP_3-induced contraction were unaffected (Fig. 5 and Table I). Two isotype-matched antibodies, one to a specific mouse idiotype (unpublished) and the other to CD37 (MB-1) (21) were used as control antibodies. Preincubation with these control antibodies had no effect on cell length in the control state nor did they have any effect on contraction induced by either contractile peptide agonist (Table I). Preincubation of isolated permeabilized cells either with 2B4-123 hybridoma supernatant or with affinity purified antibody had the same inhibitory effect on bombesin and PKC induced contraction but had no effect on substance P or IP_3 induced contractions (Table I). In confirmatory experiments, antibody specific for a second distinct hsp27 epitope was used; preincubation of permeabilized cells with antiserum specific for an N-terminal peptides of hsp27 (residues 1-15) inhibited bombesin but not substance P induced contraction (Table I). Preincubation of permeabilized smooth muscle cells with sera from two non-immune

Table I. Percent shortening (% decrease in cell length from control) obtained in response to bombesin or substance P, after preincubation for 1 hr. with the different antisera, hybridoma supernatant, or affinity purified antibody to hsp27

	% shortening	
antibody	bombesin	substance P
Hybridoma supernatant		
anti hsp27[a] (2B4-123)	8.3 ± 7.6 (2)	38.9 ± 4.9 (2)
MS5A10[a,b]	39.6 ± 1.0 (3)	36.2 ± 2.4 (3)
Affinity purified monoclonal		
anti hsp27[c] (2B4-123)	0.5 ± 1.9 (4)	35.6 ± 1.4 (4)
MB1[c]	38.4 ± 3.0 (3)	38.1 ± 3.3 (3)
Sera		
anti hsp27c[d] (123-137)	(-)2.1 ± 10.5 (2)	33.1 ± 1.4 (2)
anti hsp27n[e] (1-15)	17.5	38.7
NMS2[f]	33.6 ± 0.5 (2)	38.6 ± 1.9 (2)
NMS1[f]	33.1 ± 0.6 (2)	31.3 ± 2.1 (2)

Permeabilized cells were incubated with antibody for 1 h and bombesin or substance P induced contraction was measured as described (Figure 5).

[a]1:100 - 1:200 dilution
[b]isotype matched monoclonal antibody to a specific mouse idiotype
[c]0.2 μg/ml
[d]antisera to hsp27 peptide 2 (residues 123-137) conjugated to thyroglobulin
[e]antisera to hsp27 peptide 1 (residues 1-15) conjugated to thyroglobulin
[f]normal mouse sera

mice had no effect on contraction induced by either contractile peptide agonist. These data strongly suggest that hsp27 plays a role in bombesin/PKC but not substance P/IP_3 -induced contraction of smooth muscle.

Discussion

No specific function has previously been identified for hsp27 which is encoded by at least one member of a four gene family (22). The heat shock proteins comprise several families of proteins whose expression is greatly increased in response to heat shock or stress. Specific functions outside the heat shock response have been identified for members of the heat shock protein families (23) including roles in protein transport, oligomer assembly (24) and facilitation of proper folding of proteins. (25). Phosphorylation of hsp27 has been shown to increase in response to diverse stimuli including phorbol esters and calcium ionophores (14, 15, 18). The degree of phosphorylation varies in response to different stimuli (14, 18). Phorbol ester induced phosphorylation of hsp27 in rat myoblasts is mediated by protein kinase C (15) and in other tissues phosphorylation is mediated by alternative kinases in response to agents such as tumor necrosis factor (26, 27). Protein kinase C activation with phorbol ester is known to generate and maintain vascular smooth muscle tone in the absence of detectable elevation in [$Ca^{++}i$], (28) in the presence of low levels of myosin light chain phosphorylation (29), and in the absence of elevated Ca^{++} levels required to support myosin light chain mediated smooth muscle contraction (30).

The C-terminal domain of hsp27 is conserved (22) and bears considerable homology to lens alpha crystalline, a highly polymeric protein. Hsp27 forms higher order aggregates of 200 kD to greater than 2mD in response to various stress factors (31) and it has been suggested that the C-terminal domain is involved in formation of these high order aggregates. Hsp27 is localized in the cytosol of unstressed Hela cells (31) but has not been associated with any distinct structures. Specific phosphorylation sites on hsp27 have not been identified in smooth muscle although serine residues are phosphorylated in HeLa and mammary carcinoma (15). In turkey gizzard, a 25kD inhibitor of actin polymerization was identified as a low molecular weight heat shock protein (32).

The data support the hypothesis that hsp27 is integrally involved in sustained PKC mediated contraction seen in rectosigmoid muscle. Preincubation with the monoclonal antibody to hsp27 (2B4-123) selectively inhibited bombesin or PKC induced contraction, but not contraction induced by substance P or IP_3. The inhibition of the bombesin induced contraction does not seem to be due to an interference of the mAb with the binding of bombesin to its receptor, rather to an interference with the mechanism of the sustained contraction itself, since the preincubation with the antibody also inhibited the sustained contraction induced by exogenous PKC. Preincubation with the mAb did not inhibit the transient contraction induced by substance P or by exogenous IP3. Furthermore, the mAb does not crossreact with myosin light chain as determined by immunoblotting. Therefore, hsp27, or a cross reactive protein plays an integral role in the orientation or activation of the contractile machinery necessary to maintain a sustained contraction in gastrointestinal smooth muscle. Our data support a novel physiological role, other than seen in response to thermal stress, for the low molecular weight heat shock protein hsp27 as an important intermediate in sustained contraction of smooth muscle induced by bombesin and PKC. The finding is of considerable interest and has great implication in other types of smooth muscle (vascular, tracheal) where sustained contraction is of physiological and pharmacological importance.

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