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Cellular Pathways in Agonist-induced Gallbladder Muscle Contraction in the Cat

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ABSTRACT

Cholecystokinin octapeptide (CCK-8), acetylcholine (ACh) and KCl caused a dose dependent contraction in muscle cells enzymatically digested from cat gallbladder. Maximal contraction was obtained at concentration of 10^{-9} M for CCK-8, 10^{-5} M for ACh and 20 mM for KCl. CCK-8 induced contraction was unaffected in calcium free physiological salt solution (PSS) and was completely blocked by strontium substitution for calcium ($p<0.001$). In contrast, KCl evoked contraction was blocked in calcium free PSS ($p<0.01$) but was unaffected by strontium replacement of calcium. The contraction elicited by ACh was only slightly reduced in calcium free PSS ($p<0.05$) and was unaltered by strontium. Muscle cells permeabilized with saponin contracted in response to inositol 1,4,5-trisphosphate (IP_3) and CCK-8. The contraction was blocked by the calmodulin antagonist CGS 9343B ($p<0.001$), whereas heparin completely blocked the effect of IP_3 ($p<0.001$). The protein kinase C (PKC) antagonist H7 had no effect on either agonist. We conclude that CCK-8 induced gallbladder muscle contraction is mediated by IP_3 dependent intracellular calcium release from intracellular stores and a calmodulin dependent pathway; ACh may utilize both extracellular and intracellular calcium. KCl causes muscle contracrion through influx of extracellular calcium and a calmodulin independent machanism.

Key Words: Gallbladder muscle cells, Inositol 1,4,5-triphosphate, Protein kinase C, Calcium, Cholecystokinin octapeptide

INTRODUCTION

The gallbladder is tonically contracted in vivo or in vitro. The tone is maintained through cholinergic input and myogenic mechanisms. In the postprandial state the gallbladder contracts in response to a rise in the circulating levels of cholecystokinin (CCK) (Wiener *et al.*, 1981). In the cat CCK causes gallbladder contraction by stimulating the relaeses of ACh from presynaptic sites and by direct muscle action (Behar and Biancani, 1980).

The direct muscle action, which can be demonstrated in vivo and in vitro in muscle strips (Behar and Biancani, 1980; Yau *et al.*, 1973), is mediated by release of calcium from intracellular stores (Lee *et al.*, 1989). This conclusion is based on the finding that the contraction evoked by CCK is unaffected by the removal of extracellular calcium and is completely blocked by strontium replacement for calcium; strontium can replace the functional role of extracellular calcium in supporting contraction mediated by extracellular calcium influx, but does not support contraction mediated by release of calcium from store sites (Baba *et al.*, 1985;

Biancani *et al.*, 1987; Hotta and Tsukui, 1968; Yasuda and Sakai, 1984). Strontium is readily taken up by the endoplasmic reticulum but it probably is not easily released (Somlyo and Somlyo, 1971). Thus substitution of strontium for calcium results in inhibition of contraction mediated by release of intracellular calcium. In contrast, KCl evoked contraction is unaltered by strontium but it is blocked by the absence of extracellular calcium. Potassium is known to depolarize muscle cells and cause contraction by increasing calcium influx into the cells. ACh appears to utilize both extra and intracellular calcium since its actions are not affected by strontium or by zero calcium in the media. These results are similar to the mechanisms of CCK evoked amylase secretion in the exocrine pancreas (Gardner and Jensen, 1987; Mitchell, 1975).

The present studies were designed to characterize further the intracellular pathways utilized by CCK-8 during contraction of enzymatically digested muscle cells from the cat gallbladder. The use of enzymatically dissociated smooth muscle cells allows to characterize more precisely myogenic effects, and by the use of cells permeabilized by brief exporsure to saponin, allows the use of intracellular messengers, such as IP_3, which cannot diffuse across the plasma membrane.

MATERIALS AND METHODS

Adult cats of either sex, weighting between 3 and 5 kg, were used. The animals were anesthetized with intramuscular ketamine hydrochloride (30 mg/kg) and sacrificed with intraperitoneal pentobarbital sodium (15 mg/kg), and then the abdomen was opened with midline incision. The commom bile duct was clamped and each gallbladder with its cystic duct was removed and placed in a dissecting pan containing ice-cold Krebs buffer solution continuously aerated with 95% O_2-5% CO_2. The bile was removed carefully with a syringe so as to avoid staining the muscle, and the cavity was rinsed by repeated filling and emptying with Krebs buffer solution (its composition in mM was 116. 6 NaCl, 3.4 KCl, 21.9 $NaHCO_3$, 1.2 NaH_2PO_4, 2. 5 $CaCl_2$, 1.2 $MgCl_2$, and 5.4 glucose). Under the dissecting microscope the outer serous membrane and inter mucosal layer were carefully peeled off, and the thin muscle layer obtained was minced into 2×2 mm squares with blade block.

Single cell isolation

Cells were isolated with a modified method described previously for esophageal, gastric and intestinal smooth muscle cells (Ghosh *et al.*, 1988; Guillemette *et al.*, 1989). First, the muscle squares were digested in N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES)-buffered physiological solution, containing 0.1% (150 unit/ml) collagenase type II (Worthington Biochemicals, Freehold, NJ) under aeration with 100% O_2 at 31℃ around 1.5~2 hours. The HEPES buffer solution contained the following in mM: 114.7 NaCl, 5.6 KCl, 2.1 KH_2PO_4, 1.9 $CaCl_2$, 0.6 $MgCl_2$, and 11.1 glucose. The buffer contained also 0.08 mg/ml soybean trypsin inhibitor (Worthington Biochemicals, Freehold, NJ) and 2% (v/v) Eagle essential amino acid supplement without l-glutamine (100×, Whittaker Bioproducts, Walkersville, MD), and adjusted pH to 7.4 with 1 N NaOH at 31℃. At the end of digestion period, the tissue was placed over 450 μm Nitex filter, rinsed with 150 ml of collagenase-free HEPES buffer to remove any trace of collagenase, and then resuspended in collagenase free-HEPES buffer to allow the dissociation of the muscle cells. These cells were allowed to disperse spontaneously for 25~50 min at 31℃ with 100% O_2, thereafter, harvested by filteration through 450 μm Nitex filter.

Permeabilization of smooth muscle cells

At the end of digestion period, the tissue was also placed over 450 μm Nitex filter, rinsed with 60 ml of cytosolic buffer resembling cytosol which had following composition (in mM): 20 NaCl, 100 KCl, 25 $NaHCO_3$, 0.96 NaH_2PO_4, 0.48 $CaCl_2$ and 1 EGTA. The meduim contained 2% bovine serum albumin. The pH was adjusted to 7.2 and maintained by equilibrating with 95% O_2-5% CO_2 at 31℃. And then half di-

gested tissue was immediately resuspended in cytosolic buffer and replace at 31℃ with 95% O_2-5% CO_2 to dissociate the cells for 30 min to 1 hours. After dissociation was completed, the dispersed muscle cells were harvested by filtering through a 450 μm Nitex filter and equilibrated for about 15 min at 31℃. The harvested cells were permeabilized by exposure to saponin which was added at the concentration of 75 μg/ml. After 3 min incubation period the cells were filtered through a 10 μm Nitex filter using gentle suction and resuspended the modified buffer without saponin. The modified cytosolic buffer contained 1.5 mM ATP as an energy source and a regenerating system consisting of 5 mM creatine phaphate and 10 unit/ml of creatine phosphokinase to keep the concentration of ATP constant. Antimycin A (10 μM) was also included to prevent substrate oxidation. Then, 0.25 ml aloquets of the cell solution were added to siliconized glass tubes containing appropriate contractile agonists in order to obtain dose response relationships. The cells were allowed to react for 30 sec which was terminated by addition of acrolein at a 1.0% final concentration. For the measurement of control cell length, the contractile agents were substituted for an equivalent volume of HEPES buffer. All the glassware utilized in this experiment was rinsed in a 1% silicon solution to prevent the cells from adhering to the glass.

The following drugs were used: cholecystokinin octapeptide (CCK, sulfate 26~33, Bachem, Torance, CA), acetylcholine chloride and heparin (low M.W. 4,000~6,000) (Sigma Chemical, St. Louis, MO), CGS 9343B (1,3-dihydro-1-[1-((4-methyl-4H,6H-pyrrolo[1,2-a]-[4,1]benxazepin-4-yl)methyl)-4-piperindinyl]-2H-benzinidazol-2-one maleate, Ciba-Geigy, Summit, NJ), H-7 (1-(5-isoquinolinsulfonyl)-2-methylpiperazine dihydrochloride, Seikagaku Kogyo Co., Tokyo, Japan).

Measurement of contraction

Contraction was measured in cell suspensions as described previously (Biancani *et al.*, 1987; Bitar and Makhlouf, 1986). Thirty to fifty consecutive intact cells in microscopic field were measured through a phase contrast microscope (Carl Zeiss), a TV camera (model WV-1550, Panasonic, Secaucus, NJ), and a TV screen (model WV-5410, Panasonic). The camera was connected to a video microscaler (model IV-550, FOR-A, West Netwon, MA). The microscaler superimposes two vertical and one horizontal line onto the TV screen. Cells were oriented along the horizontal line on the TV screen by rotating the slide on a rotating microscope stage, and the cell length was obtained by placing the two vertical lines. The contractile responses were expressed as the percentage shortening of the cell length from corresponding control.

Statistical analysis

All data are expressed as means±S.E. of the mean. Significant differences were evaluated using paired or unpaired Student's t-test.

RESULTS

CCK-8, ACh and KCl-induced contraction

CCK-8, ACh and KCl caused dose dependent gallbladder muscle cell contraction. Figure 1A shows contraction in response to increasing concentrations of CCK-8. Maximal contraction (28±2%) was obtained with 10^{-9} M CCK-8. The maximal response to ACh was 28±2% at 10^{-5} M (Fig. 1B). Maximal response to KCl was 24±2% at 20 mM (Fig. 1C)

Effect of Ca^{2+}-free meduim and strontium on agonist-induced contraction

The contraction induced by a maximal CCK-8 was unaffected by incubation of the gallbladder muscle cells in calcium free PSS containing 2 mM EGTA (Fig. 2). Maximal KCl-induced contraction was completely abolished ($p<0.01$) by incubation in this PSS, while the ACh induced contraction was slightly but significantly diminished ($p<0.05$). In contrast, when calcium was replaced by 4 mM strontium, the contraction induced by CCK-8 was almost completely blocked ($p<0.05$) while the contractions induced by ACh and KCl were unaffected (Fig. 3).

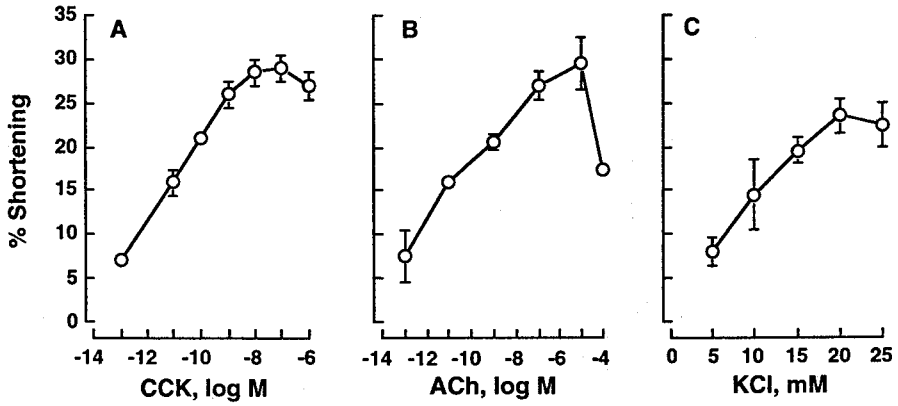

Fig 1. Effects of increasing concentrations of CCK-8 (A), ACh (B) and KCl (C) on intact gallbladder muscle cells in normal HEPES buffer (calcium: 2.5 mM). Maximal muscle contraction was attained at the concentration of 10^{-9} M CCK-8, 10^{-5} M ACh and 20 mM KCl. Valvues are means ±SE of four experiments.

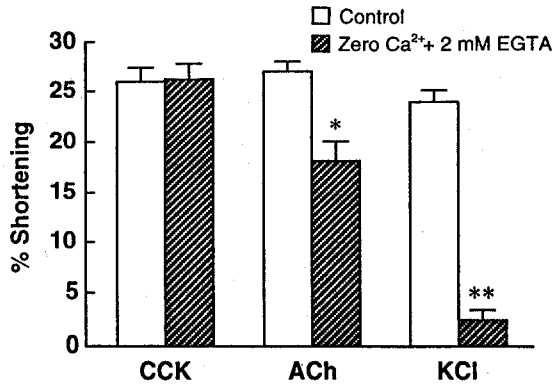

Fig. 2. Effect of calcium-free medium (zero Ca^{2+} + 2 mM EGTA) on the response of intact gallbladder muscle cells to maximal concentrations of CCK-8 (10^{-9} M), ACh (10^{-5} M) and KCl (20 mM). Values are means±SE of 4 paired experiments. There were no differences in the muscle contraction in either media for CCK-8, slight but significant reducion in the response to ACh ($p<0.05$) and marked decrease in the response to KCl ($p<0.001$).

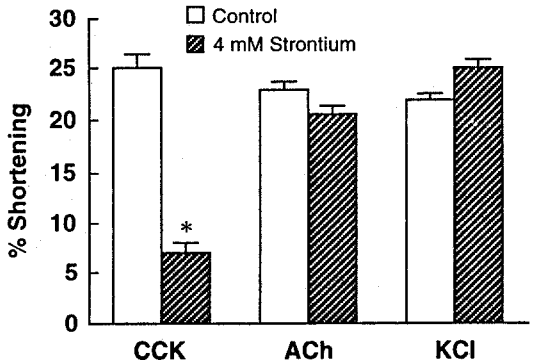

Fig. 3. Effect of strontium replacement of calcium on the response of intact gallbladder muscle cells to maximal concentrations of CCK-8 (10^{-9} M), ACh (10^{-5} M) and KCl (20 mM). Values are means±SE of four paired experiments. Strontium caused a marked block of the CCK-8 action ($p<0.001$).

CCK-8 and IP_3-induced contraction in permeabilized cells

Muscle cells permeabilized with saponin were contracted in response to increasing concentrations of CCK-8 (10^{-13} to 10^{-6} M) as much as intact cells suggesting that brief exposures to saponin did not affect significantly the number or the functional integrity of CCK receptors (Fig. 4.) The permeabilized muscle cells also contracted in a dose-dependent manner in response to increasing concentrations of IP_3 (10^{-10} to 10^{-6} M, Fig. 5). IP_3 had no effect on intact muscle

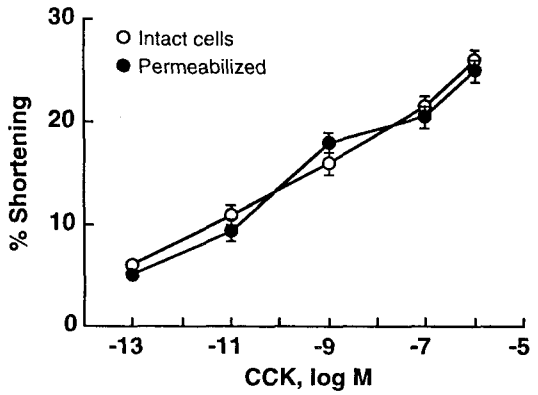

Fig. 4. Dose-response relationships of CCK-8 on intact and permeabilized gallbladder muscle cells with saponin. Values are means±SE of four paired experiments. There were no significant differences between both dose-response studies.

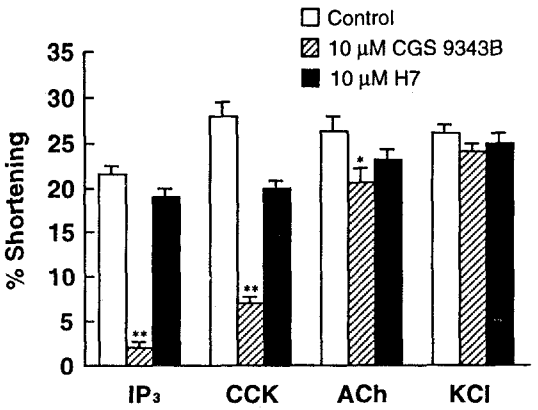

Fig. 6. Effect of the calmodulin antagonist, CGS 9343B and PKC inhibitior, H7 on the action of maximal concentractions of IP_3 (10^{-6} M), CCK-8 (10^{-9} M), ACh (10^{-5} M) and KCl (20 mM). Values are means±SE of four paired experiments. CGS 9343B blocked the actions of IP_3 and CCK-8 ($p<0.001$) but had no effect on the actions of ACh and KCl. H7 had no effect on either agonist.

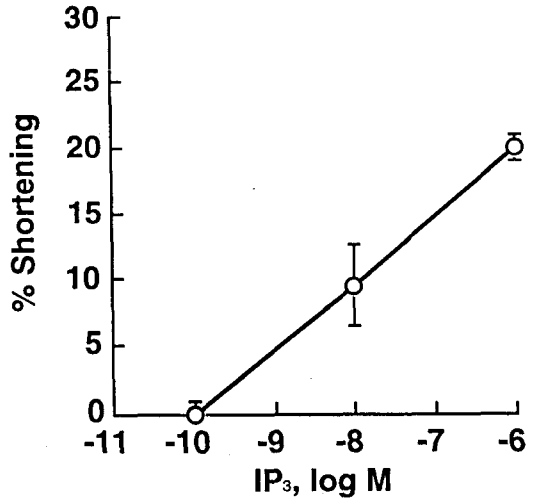

Fig. 5. Effect of increasing concentrations of IP_3 on permeabilized gallbladder muscle cells. Values are means±SE of four paired experiments.

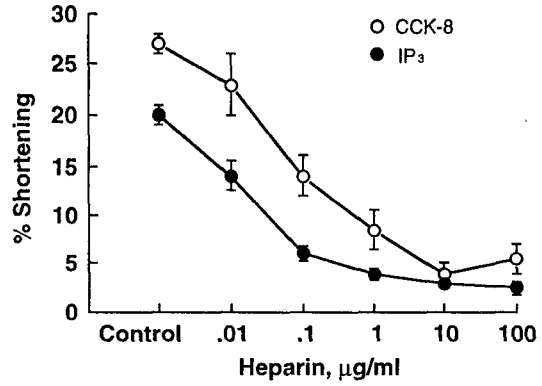

Fig. 7. Effect of increasing concentrations of heparin on the actions of a maximal concentration of CCK-8 (10^{-9} M) and IP_3 (10^{-6} M) in permeabilized gallbladder muscle cells. Values are means±SE of four paired experiments. Heparin almost completely blocked the actions of CCK-8 ($p<0.001$) and IP_3 ($p<0.001$)

cells since it cannot diffuse through the cell membrane.

Effect of CGS 9343B and H7 on agonist-induced contraction

The calmodulin antagonist CGS 9343B at the concentration of 10^{-5} M (Fig. 6) almost completely blocked the contraction caused by 10^{-6} M IP_3 ($p<0.01$) and by a maximal concentration of CCK-8 of 10^{-9} M ($p<0.01$). It slightly reduced the response to 10^{-7} M acetylcholine ($p<0.05$) and had no effect on the muscle response to a maximal concentration of KCl (20 mM). However, H7 had no effect on either agonist-induced contraction.

Effect of heparin on agonist-induced contraction in saponified cells

The magnitude of the contraction induced by a maximal concentration of CCK-8 (10^{-9} M) or IP_3 (10^{-6} M) was gradually reduced by increasing concentrations of heparin and was almost completely blocked at a concentration of 10 μg/ml of heparin (Fig 7. $p<0.001$).

DISCUSSION

CCK-8, ACh and KCl cause dose dependent contraction of gallbladder muscle cells (Lee *et al.*, 1989). CCK-8 contracts the muscle cells by releasing calcium from storage sites since its actions were not altered by the absence of extracellular calcium. This view is supported by the finding that KCl was unable to evoke muscle contraction under the same experimental conditions: potassuim is known to cause muscle contraction by depolarizing cell membranes and inducing calcium influx from the extracellular space.

It is also consistent with the finding that calcium replacement with strontium blocked the CCK-8 evoked contraction but had no effect on potassium induced contraction. Strontium can replace calcium in supporting contraction mediated by extracellular calcium influx, but does not support contraction mediated by release of calcium from intracellular stores (Arner *et al.*, 1983; Biancani *et al.*, 1987). Strontium is taken up in the endoplasmic reticulum but is not readily released (Somlyo and Somlyo, 1971).

Thus, substitution of stontium for calcium results in inhibition of processes that are mediated by release of intracellular calcium. In contrast, ACh can induce gallbladder muscle contraction by utilizing both extracellular calcium and calcium from storage sites. It is conceivable that ACh both increases calcium influx and releases calcium from intracellular stores by acting on separate muscarinic receptors (Barnard, 1988). Each receptor subtype may be linked to distinct G-proteins, one activating phospholipase C and the other causing the opening of a voltage operated calcium channel (Brown and Birnbaumer, 1988; Rosenthal and Schultz, 1987). None of the actions evoked by the three agonists were significantly affected by H7, a specific PKC inhibitor (Tomaoki *et al.*, 1986). H7 however relaxes the spontaneous tone maintained by gallbladder muscle strips (Lee *et al.*, unpublished observations).

It has been shown that IP_3 is a second messenger mediating release of calcium from intracellular sites by activating specific receptors on the endoplasmic reticulum (Bitar *et al.*, 1986; Hashimoto *et al.*, 1985; Hirata *et al.*, 1985). Thus, the finding that IP_3 can contract permeabilized muscle cells is an additional demonstration that these cells contain sufficient calcium stores. In contrast esophageal muscle cells which do not utilize calcium stores do not contract in response to IP_3 (Biancani *et al.*, 1987). The findings that both CCK-8 and IP_3 induce muscle contraction by utilizing calcium from intracellular stores and that this contraction is blocked by heparin, strongly suggest that the CCK evoked contraction is mediated by IP_3. Heparin blocks specifically the IP_3 activated calcium release mechanisms without affecting other calcium transport processes such as the ATP-dependent Ca^{2+} pump or the CCK-8 actions were also blocked by the calmodulin antagonist CGS 9343B which is highly specific for calmodulin mediated cellular processes since it does not alter the actions mediated by protein kinase A and PKC (Norman *et al.*, 1987). These results suggest that CCK-8 causes gallbladder muscle contraction by utilizing an IP_3 linked pathway that releases calcium from

storage sites which in turn activates calmodulin. Calmodulin appears to specifically mediate metabolic processes that utilize calcium released from storage sites, since CGS 9343B did not affect the muscle contraction elicited by KCl which causes influx of extracellular calcium. This calmodulin antagonist affected only slightly the muscle contraction induced by ACh perhaps because this agonist can utilize more than one intracellular pathway.

In summary, the data suggest that CCK evokes cat gallbladder muscle contration by receptor mediated activation of IP_3 and release of calcium from intracellular stores, resulting in activation of calmodulin. K^+ induces muscle contration by inducing calcium influx from the extracellular space and appears to be mediated by pathways other than IP_3-calmodulin or diacylglycerol (DAG)-PKC. The data also suggest that ACh causes gallbladder contraction via a dual pathway. Like CCK-8 it utilizes IP_3-induced calcium release and a calmodulin dependent pathway, and it utilizes extracellular calcium influx and different intracellular mechanisms. None of these agonists seem to activate the DAG-PKC pathway since their actions were unaltered by H7. Thus, they suggest the existence of at least two cellular pathways that mediates agonist induced contraction. One that utilizes IP_3, stored calcium and calmodulin; the other mediated by influx of extracellular calcium with still undetermined second messengers.

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=국문초록=

고양이의 담낭근 수축에 있어서 세포내 기전

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고양이 담낭근에서 효소학적으로 분리한 평활근 세포는 cholecystokinin octapeptide (CCK-8), acetylcholine (ACh) 및 KCl에 의하여 용량에 의존하여 수축하였다. 이들 효현제 (CCK-8, ACh 및 KCl)에 의한 평활근 세포의 최대수축은 각각 10^{-9} M, 10^{-5} M 및 20 mM 농도에서 야기되었다. CCK-8에 의하여 야기되는 이들 평활근 세포의 수축은 HEPES 완충액에 Ca^{2+}을 제거시킴에 의하여 영향을 받지 아니하였으나, Ca^{2+} 대신에 strontium을 첨가시켰을때 수축반응이 완전하게 억제되었다 ($p<0.001$). 이와는 반대로 KCl에 의한 수축반응은 strontium 치환에 의하여 영향을 받지 아니하고 HEPES 완충액에 Ca^{2+}을 제거시킴에 의하여 억제되었다 ($p<0.01$). ACh에 의하여 야기되는 수축반응은 세포 외액의 Ca^{2+}을 제거시킴에 의하여 중등도의 억제반응이 야기되었으나 ($p<0.05$) strontium에 의하여 영향을 받지 아니하였다. Saponin으로 세포 투과성 변동을 야기시킨 근세포에서 inositol 1,4,5-trisphosphate (IP_3)와 CCK-8은 수축반응을 일으켰고, 이러한 수축반응은 calmodulin 길항제인 CGS 9343B에 의하여 차단되었으며 ($p<0.001$), heparin은 CCK-8 및 IP_3의 작용을 완전하게 봉쇄하였다 ($p<0.001$). 그러나 이러한 수축반응에 있어서 protein kinase C 길항제인 H7은 아무런 작용을 나타내지 못하였다. 이러한 결과로 볼 때 CCK-8에 의하여 야기된 고양이 담낭근 세포의 수축반응은 IP_3에 의하여 세포내 저장소로 부터 유리된 Ca^{2+}과 calmodulin에 의존적인 과정에 의하여 매개되어 지는 것으로 생각된다. 또한 ACh는 세포외액의 Ca^{2+} 뿐만 아니라 세포내 저장소의 Ca^{2+} 모두를 이용하며, KCl은 전적으로 세포외액의 Ca^{2+}에 의존적인 형태로 calmodulin과는 무관하게 고양이 담낭근 세포의 수축반응을 야기시키는 것으로 사료된다.