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Contribution of different phospholipases and arachidonic acid metabolites in the response of gallbladder smooth muscle to cholecystokinin

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Abstract

Guinea pig gallbladder muscle strips were used to investigate the contribution of different sources of diacylglicerol (DAG) in the cholecystokinin (CCK)-induced contraction. The involvement of arachidonic acid (AA) in this response was also investigated. Three distinct pathways for DAG production were investigated with specific phospholipase (PL) inhibitors. U-73122 (10 μM) was used for inhibition of phosphoinositide-specific-PLC (PI-PLC), D-609 (100 μM) for phosphatidylcholine specific-PLC (PC-PLC), and propranolol (100 μM) for phospholipase D (PLD). Separate or combined inhibition of each of these enzymes showed that the CCK-induced output of DAG involves the parallel activation of each of these phospholipases. Thus, after inhibition of a PL subtype, the remaining subtypes were able to functionally compensate in mediating CCK-induced contraction. Inhibition of AA production via DAG-lipase or phospholipase A_2 (PLA_2) was accomplished using RHC-80267 (40 μM), mepacrine (100 μM) and 4-BPB (100 μM). These inhibitors diminished contractile response, indicating that AA is an important modulator of CCK-induced contraction. Indomethacin (10 μM) and nordihydroguaiaretic acid (NDGA, 100 μM), which inhibit subsequent steps in AA metabolism through the cyclooxygenase and 5-lipooxygenase pathways, also inhibited contractions. Taken together, these results show that CCK redundantly activates PC-PLC, PI-PLC and PLD, to produce DAG, which in turn stimulates PKC and provides a substrate for the generation of AA. $sPLA_2$ is also a source of AA, whose metabolites are, in part, responsible for determining the magnitude of the CCK-evoked contraction.

Keywords: PI-PLC; PC-PLC; PLD; PLA_2; Cyclooxygenase; 5-Lipoxygenase

1. Introduction

CCK is a major physiological hormone that regulates gallbladder contraction and emptying of bile during the intestinal phase of the postprandial state. In the gallbladder, CCK-induced contraction is mediated by interaction with the PTx-sensitive $G_{i\alpha3}$ protein, which activates the PI-PLC [1]. PI-PLC catalyzes the hydrolysis of phosphatydilinositol 4,5-biphosphate (PIP_2) and yields two messengers, IP_3 and DAG. IP_3 releases Ca^{2+} from internal stores, which is followed by influx of external Ca^{2+} [2]. Ca^{2+} binding to CaM enhances the activity of MLCK, leading to the phosphorylation of the 20 kDa light chains of myosin and triggering the myosin ATPase activity and the subsequent smooth muscle contraction [3,4].

The DAG formed as part of this pathway also plays an important messenger function. DAG is a lipophilic molecule that remains within the plasmalema and activates several isoforms of the protein kinase-C (PKC) family [5]. These kinases regulate smooth muscle contraction by the phosphorylation of proteins other than myosin [6,7]. Other phospholipases can function as alternate, parallel sources of DAG. For example, PC-PLC acts to produce phosphocholine and DAG, while PLD cleaves PC to produce PA, later dephosphorylated by an additional enzyme (PA-PH) to produce DAG [8,9] (see Fig. 1). The relative contributions of these parallel PL pathways

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Abbreviations: AA, arachidonic acid; ACh, acetylcholine; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CaM, calmodulin; CCK, cholecystokinin; DAG, diacylglicerol; EC_{50}, concentration producing 50% of the maximal response; E_{max}, maximal response; K–HS, Krebs–Heinseleit solution; IP_3, inositol 1,4,5-trisphosphate; MLCK, myosin light chain kinase; NDGA, nordihydroguaiaretic acid; PA, phosphatidic acid; PA-HA, phosphatidic acid phosphohydrolase; PC, phosphatidylcholine; PC-PLC, phosphatidylcholine specific-PLC; PL, phospholipase; PLA_2, phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; $sPLA_2$, secreted phospholipase A_2; PLC, phospholipase C; PI-PLC, phosphoinoisitide specific-PLC; PLD, phospholipase D; PKC, protein kinase C.

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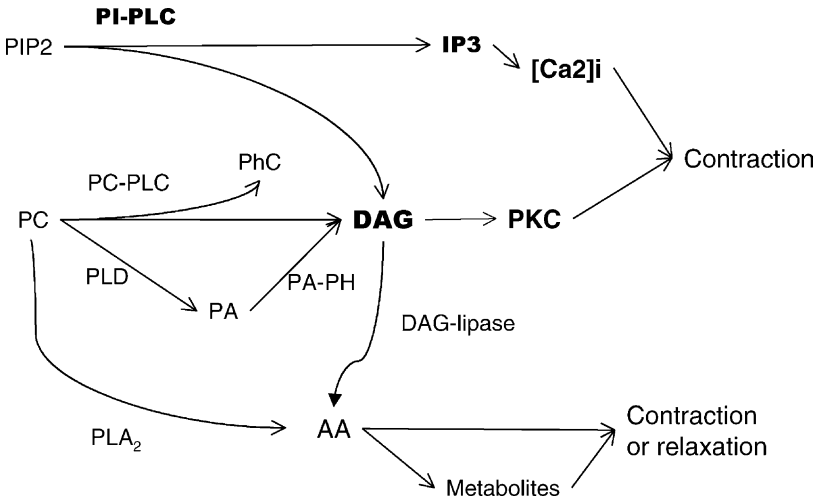

Fig. 1. Main intracellular pathways leading to DAG and AA output. The main effects of some messengers are also indicated. The known pathways activated by CCK to induce gallbladder contraction are shown in bold.

to CCK-induced DAG production and contractile modulation are unknown in the gallbladder.

In addition, DAG can be hydrolyzed by DAG-lipase to yield AA [10] which can also be formed from PC by PLA_2 [9]. AA has multiple cellular effects either directly [11] or as a precursor to prostaglandins, leukotrines and thromboxanes [12–16]. These metabolites have been shown to play an active role in gallbladder disease [17], and increases in prostaglandin synthesis in response to CCK have been described in cultured myocytes from bovine gallbladder [18]. However, the actions of AA and its metabolites, in modulating gallbladder smooth muscle contraction have not been studied. The present investigation was designed to test the relative importance of several PL pathways in mediating CCK-induced gallbladder contraction, and to determine the contribution of AA to this response. Our results indicate that PI-PLC, PC-PLC, and PLD act as redundant pathways in the CCK-induced production of DAG. The DAG in turn, activates PKC and leads to the generation of AA and its metabolites. AA output plays a key role in CCK-mediated response but it lacks direct contractile effects.

2. Materials and methods

2.1. Animals and tissue preparation

Gallbladders were isolated from 300 to 450 g male guinea pigs following cervical dislocation and exanguination and immediately placed in cold K–HS of the following composition (mM): NaCl 113, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and D-glucose 11.5 at pH 7.35. All procedures were reviewed and approved by the Office of Animal Care Management at the University of Extremadura.

The gallbladder was opened by cutting along the longitudinal axis and trimmed of any adherent liver tissue. After washing with the nutrient solution to remove any biliary component, the mucosa was scraped off with a cotton tip and the gallbladder was cut into strips along the longitudinal axis, each strip measured approximately 3 mm × 10 mm. Typically, four strips were obtained from each guinea pig gallbladder. Each strip was placed vertically in a 10 mL organ bath filled with the nutrient solution maintained at 37° and gassed with 95% O_2 and 5% CO_2. Isometric contractions were measured using force displacement transducers connected to a MacLab system consisting of a MacLab hardware unit (MacLab/4e, ADInstruments Pty Ltd) and Chart version 3.6.8 software, (ADInstruments Pty Ltd) which runs on a Macintosh computer.

The strips were placed under an initial resting tension equivalent to a 1.5 g load and allowed a 60 min period for equilibration, during which time the nutrient solution was changed every 20 min. The muscle length corresponding to the optimal preload was then determined by increasing the length of each strip in increments of 1 mm until a maximal response to ACh (10 μM) was achieved. The optimal preload muscle length was maintained throughout the duration of the experiments. Finally, the gallbladder strips were washed thoroughly and the experimental protocols commenced.

2.2. Experimental plan

To determine the contribution of the different pathways implied in the CCK-induced gallbladder smooth muscle contraction a protocol of cumulative concentration–response curves for CCK was designed.

The cumulative concentration–response curve commenced with the lowest concentration of CCK and the subsequent doses were not added until the contractile response to the agonist became constant. After the maximal response was reached (about 40 min), 1 hr was required for washing out and returning to the resting

tension. Then, the muscle strips were incubated for 20 min with the antagonists and the cumulative concentration–response curve was repeated.

To test whether CCK-8-induced contractions were a direct effect of the peptide we used atropine (1 μM) and tetrodotoxin (TTX, 1 μM) as blockers of muscarinic receptors and neural activity, respectively.

To establish the sources of DAG in the gallbladder and determine what phospholipases can lead to DAG production [8,19,20] we used specific PL inhibitors. U-73122 (10 μM) was used for inhibition of PI-PLC and D-609 (100 μM) for PC-PLC (these doses are in the range reported in previous studies [8,21,22]). DAG is also produced from PC by the sequential action of PLD and PA-PH. Propranolol, which inhibits PA-PH, has been shown to be an effective blocker of DAG production through PLD pathway when used at high concentrations (100 μM) [8]. As the inhibitors had negligible effects when tested separately, we tested higher concentrations (30 μM for U-73122 and 200 μM for D-609) on the 10 nM CCK-8-induced contraction, but no additional effect was detected. To avoid uncontrolled side effects, we used the referenced doses. Preliminary results showed that higher doses of propranolol (300–500 μM) caused a great reduction in both CCK and KCl-induced contraction, indicating that non-desirable side effects may lead to the inhibition.

To explore the CCK-8-induced AA production we used RHC-80267 (40 μM), mepacrine (100 μM), 4-BPB (100 μM) and AACOCF3 (100 μM), inhibitors of DAG-lipase, sPLA2 and cPLA$_2$, respectively.

The role of AA metabolites in CCK-8-induced response was assessed by using indomethacin (10 μM) and NDGA (100 μM), inhibitors of cyclooxygenase and 5-lipooxygenase, respectively.

To test the contractile response to AA and to characterize this response, single concentrations of AA were used, as desensitization of the tissue was observed. To study the intracellular pathways stimulated by AA, indomethacin, NDGA, methoxyverapamil, and the CaM antagonist W-7 (500 μM) were added to the organ bath 20 min before the second addition of AA. To generate reproducible responses to two doses of AA a resting period of at least 6 hr was needed.

2.3. Drugs and chemicals

ACh chloride, AA (5,8,11,14-eicosotetranoic acid), atropine sulfate, 4-BPB (4-bromophenacyl bromide), cholecystokinin (26–33) (CCK-8) sulfated, ethylene glycol-bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetracetic acid (EGTA), indomethacin, mepacrine (6-chloro-9(4-diethylamino)-1-methyl-butyl)amino-2-methoxyacridine, methoxyverapamil, NDGA, propranolol, tetrodotoxin and W-7 (*N*-(6-aminohexyl)-chloro-1-naphthalenesulfonamide) were from Sigma, and AACOCF$_3$, D-609, GF109203X, RHC-80267 and U-73122 were from Calbiochem.

Stock solutions of arachidonic acid were dissolved in ethanol and stored at −20° until use. AACOCF$_3$, 4-BPB, indomethacin, GF109203X, NDGA, RHC-80267 and U-73122 were prepared in dimethylsulfoxide (DMSO). The solutions were diluted so that the final concentration of DMSO in the organ bath was ≤0.1% (v/v). All other drugs were dissolved in distilled water.

The modified K–HS was prepared by substituting EGTA (0.5 mM) for $CaCl_2$ to obtain the Ca^{2+}-free K–HS.

2.4. Statistical analysis of the data

Changes in tone in response to agonists were determined by calculating the difference between the mean tension over a 1 min period prior to adding the agonist and the mean tension over a 1 min period after reaching the maximal response. In strips that had phasic activity, about 3–4 phasic contractions occurred during the 1 min period (frequency of phasic activity: 0.053 ± 0.008 Hz); thus averaging the tension over a 1 min period allowed for an unambiguous means of analyzing the data. CCK-8-induced contractions were expressed as a percentage of the maximal response. Each concentration–response curve was analyzed to evaluate the EC_{50} by using GraphPad Prism v. 3.00 software (GradPad Software, Inc.). The arachidonic acid-induced responses were expressed in absolute values (mN) and as percentages of the response elicited by ACh (10 μM). Data are expressed as mean ± SEM. Statistical differences between means were determined by Student's *t*-test. Differences between multiple groups were tested using ANOVA for repeated measures and checked for significance using the Scheffe's *F*-test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. General characterization of CCK-induced gallbladder contraction

In an initial set of experiments, we tested whether two comparable consecutive concentration–response curves for CCK-8 could be constructed with the same gallbladder strip. In our experimental conditions, the contractile effects of CCK-8 (10 pM–1 μM) were reproducible, since both the E_{max} (29.4 ± 3.0 mN vs. 33.7 ± 3.6 mN) and the EC_{50} (5.2 ± 0.3 nM vs. 3.36 ± 0.4 nM) were similar for both curves. Therefore, to study the effects of different agents on the CCK-8-evoked gallbladder contraction, two concentration–response curves were done sequentially. CCK-8-induced contractions were not affected neither by 1 μM atropine nor by 1 μM tetrodotoxin as indicated by no significant changes in either the EC_{50} or E_{max} for both antagonists (Table 1). These results indicate that the CCK-induced contraction involves a direct effect of the peptide on gallbladder smooth muscle cells, without the

Table 1
EC_{50} and E_{max} of the CCK-8-evoked concentration–response curve under different treatments

Treatment	EC_{50} (nM)		E_{max} (mN)		N
	Absence	Presence	Absence	Presence	
Atropine (1 μM)	6.7 ± 0.3	7.1 ± 0.3	19.6 ± 1.2	23.1 ± 2.2	5
TTX (1 μM)	4.0 ± 0.8	4.0 ± 0.8	19.1 ± 2.0	20.3 ± 1.3	5
GF109203X (10 μM)	2.1 ± 0.4	10.4 ± 2.2*	22.4 ± 2.0	16.0 ± 1.3**	7
U-73122 (10 μM)	14.0 ± 0.7	3.1 ± 1.8	22.9 ± 2.8	22.8 ± 4.4	8
D-609 (100 μM)	8.2 ± 4.8	7.1 ± 0.4	26.6 ± 2.6	23.7 ± 2.4	7
Propranolol (100 μM)	16.0 ± 10.0	4.7 ± 0.9	30.1 ± 2.2	22.6 ± 2.0***	6
Propranolol + U-73122	10.0 ± 1.4	9.9 ± 1.4	32.6 ± 8.4	19.5 ± 2.4***	7
Propranolol + D-609	9.7 ± 3.6	8.1 ± 1.6	33.9 ± 1.9	19.3 ± 1.6***	6
U-73122 + D-609	5.3 ± 0.3	14.8 ± 3.4*	34.5 ± 3.2	27.0 ± 1.7*	6
U-73122 + D-609 + propranolol	17.0 ± 9.0	29.0 ± 13.0	35.5 ± 3.3	17.0 ± 2.4***	7
RHC-80267 (40 μM)	1.4 ± 0.3	10.8 ± 2.9***	23.3 ± 1.6	22.9 ± 1.8	8
Mepacrine (100 μM)	1.8 ± 0.2	11.9 ± 4.1*	30.6 ± 5.9	14.9 ± 4.8***	4
4-BPB (100 μM)	3.6 ± 1.2	17.9 ± 7.2*	23.2 ± 2.7	7.8 ± 2.0***	4
$AACOF_3$ (100 μM)	1.8 ± 0.6	1.7 ± 0.6	21.5 ± 3.8	22.1 ± 3.4	4
Indomethacin (10 μM)	3.4 ± 1.2	14.7 ± 6.4***	20.0 ± 2.1	17.3 ± 1.7	8
NDGA (100 μM)	3.3 ± 1.4	9.4 ± 2.6*	35.4 ± 0.4	28.8 ± 1.1**	4
Indomethacin + NDGA	4.9 ± 1.1	14.2 ± 5.3*	26.5 ± 1.3	20.7 ± 2.1*	5

Data represent the mean ± SEM of N experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to the value in the absence of inhibitor.

involvement of neurotransmitter release from intrinsic nerves.

To test whether CCK-induced contraction through activation of general mechanisms as CaM-dependent MLCK and PKC, we assessed the effects of the CaM-MLCK antagonist, W-7, and the PKC inhibitor GF109203X. Fig. 2 shows that W-7 completely abolished the response to CCK-8 at all concentrations assayed ($N = 4$, $P < 0.001$), while GF109203X (1 μM) shifted the CCK-8 concentration–response curve to the right (Table 1) and reduced the maximal contractile response by 27.8 ± 3.5% ($N = 7$, $P < 0.01$, Fig. 2, Table 1).

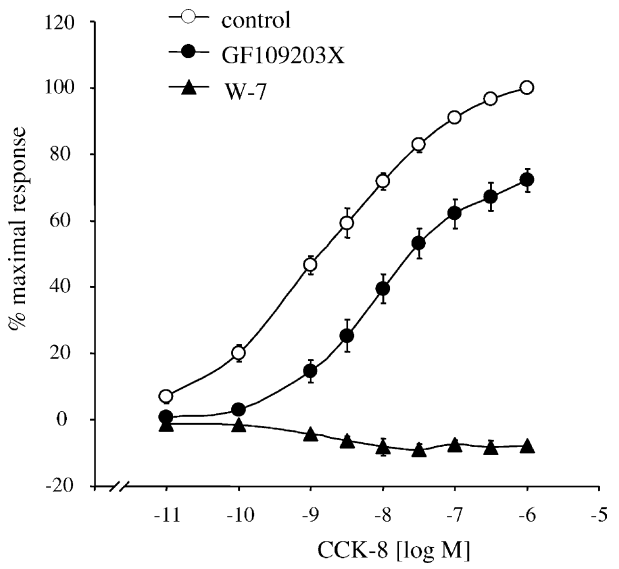

Fig. 2. Effects of pretreatment with the PKC inhibitor GF109203X (1 μM) and with the CaM inhibitor W-7 (500 μM) on CCK-8 contractile response. After performing the control curve, the inhibitors were added during 20 min and a second curve was repeated in presence of the inhibitor. Data points indicate means from the number of experiments ($N = 7$ for GF109203X and 4 for W-7, respectively) and vertical lines show SE of the mean.

3.2. DAG sources in CCK-induced gallbladder responses

The inhibition by GF109203X of CCK-8-induced contraction suggests that, in guinea pig gallbladder, DAG may be the principal activator for several isoforms of PKC and is a key mediator of CCK's contractile effect. Since the activity of several PLs can lead to DAG production (see Section 2.2) we tested the CCK-8 response in presence of selective PL inhibitors. Surprisingly, when the strips were incubated for 20 min with specific inhibitors of the PI-PLC and PC-PLC, namely U-73122 (10 μM) and D-609 (100 μM), CCK-8-evoked contraction was not altered (Fig. 3A and B, Table 1). Then, we used 100 μM propranolol to prevent DAG output through PLD sensitive pathway (see Section 2.2). However, this treatment only induced a slight decrease in the response at the highest CCK-8 concentrations (CCK 1 μM, 27.4 ± 7.7% of reduction, $N = 6$, $P < 0.001$, Fig. 3C, Table 1).

These results suggested that PLC activity and DAG production were not involved in the CCK-8-evoked contraction, which was inconsistent with the inhibitory effect that is described above for the PKC inhibitor. This discrepancy could be explained by the simultaneous production of DAG from several sources contributing to the CCK-8-evoked contraction, such that the effects of inhibiting a given PL may enhance the activation of the others and mask the role of the PL of interest. To test this, we evaluated the effects of different combinations of the PL inhibitors. Propranolol (100 μM) in combination with U-73122 (10 μM) or with D-609 (100 μM) reduced the maximal effect of the CCK-8 concentration–response

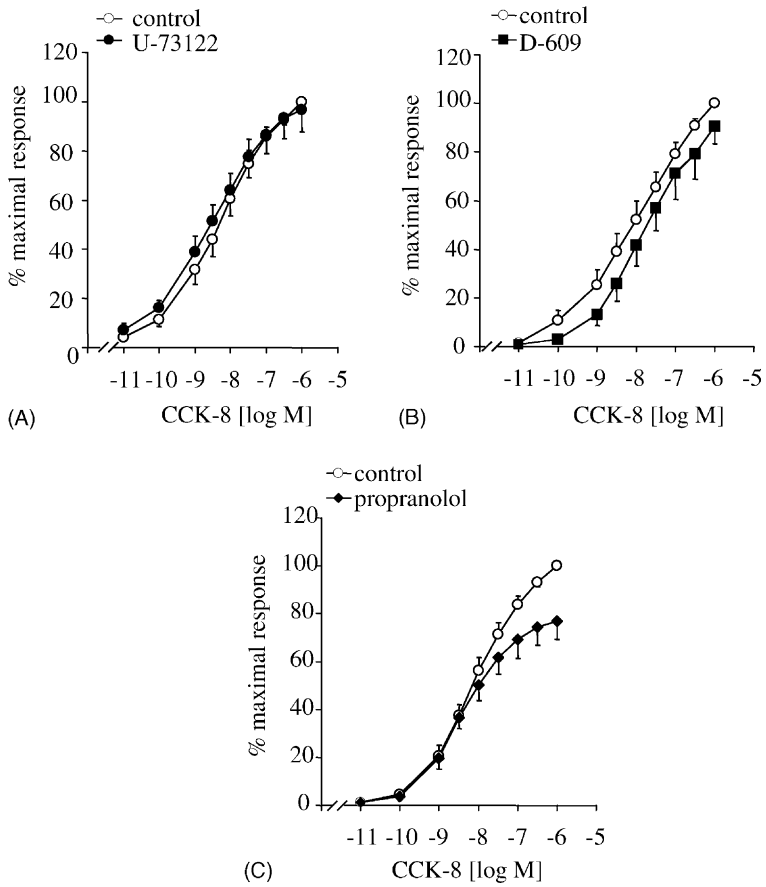

Fig. 3. (A) Effects of the PI-PLC inhibitor U-73122 (10 μM), (B) the PC-PLC inhibitor D-609 (100 μM) and (C) the PA-PH inhibitor propranolol (100 μM) on CCK-8-evoked contraction. Data points indicate means from six experiments and vertical lines show SE of the mean.

curves by $40.0 \pm 6.1\%$ ($N = 7$, $P < 0.001$, Fig. 4A, Table 1) and $43.1 \pm 3.2\%$ ($N = 6$, $P < 0.001$, Fig. 4B, Table 1), respectively. In both cases, the curves were right-shifted. Similarly, combination of the two PLC inhibitors, caused a statistically significant reduction of the maximal response ($16.9 \pm 5.1\%$, $N = 6$, $P < 0.05$, Fig. 4C, Table 1) and a rightward shift of the concentration–response. When a combination of U-73122, D-609 and propranolol was used there was a more pronounced inhibition of the responses to high concentrations of CCK-8 ($49.8 \pm 8.1\%$ inhibition, $N = 7$, $P < 0.001$, Fig. 4D, Table 1).

3.3. AA as mediator of CCK-induced contraction

To investigate whether AA contributes to the contractile effect of CCK-8, we used inhibitors of the intracellular pathways leading to AA production. Thus, when the tissue was exposed to RHC-80267 (40 μM), an inhibitor of DAG-lipase, the concentration–response curve was rightward shifted ($N = 8$, $P < 0.001$), although the maximal effect was unaffected, as shown in Fig. 5A. In contrast, both mepacrine (100 μM), a general inhibitor of PLA_2, and 4-BPB (100 μM), an inhibitor of $sPLA_2$, reduced the maximal response to CCK-8 (55.5 ± 7.1 and $63.6 \pm 10.5\%$ reduction, respectively, $N = 4$, $P < 0.001$, Fig. 5B and C, Table 1). However, AACOCF3, an inhibitor of $cPLA_2$ had no effect on the contractile response (Fig. 5D, Table 1). Together, these results demonstrate that endogenous AA is involved in the response to CCK-8.

AA is converted to prostaglandins, thromboxanes and prostacyclins by cycloxygenase, and to leukotrienes by 5′ lipoxygenase. Thus, we investigated the role of these enzymes in CCK-8-induced contraction using the cycloxygenase inhibitor, indomethacin (10 μM) and the lipoxygenase inhibitor, NDGA (100 μM). Indomethacin significantly increased the EC_{50} ($N = 8$, $P < 0.001$, Table 1), but the maximal effect was not significantly reduced (Fig. 6A, Table 1), whereas NDGA reduced the maximal response by $18.8 \pm 2.8\%$ ($P < 0.01$, Table 1) and also increased the EC_{50} ($N = 4$, $P < 0.05$, Fig. 6B, Table 1). When the tissue was treated with a combination of the two inhibitors, no additional reduction was detected ($N = 5$, Fig. 6C, Table 1).

3.4. Intracellular mechanisms involved in AA-induced gallbladder contractions

The previous results suggested that AA (directly or through its metabolites) is a contractile messenger in smooth muscle gallbladder cells. If this is true, exogenous

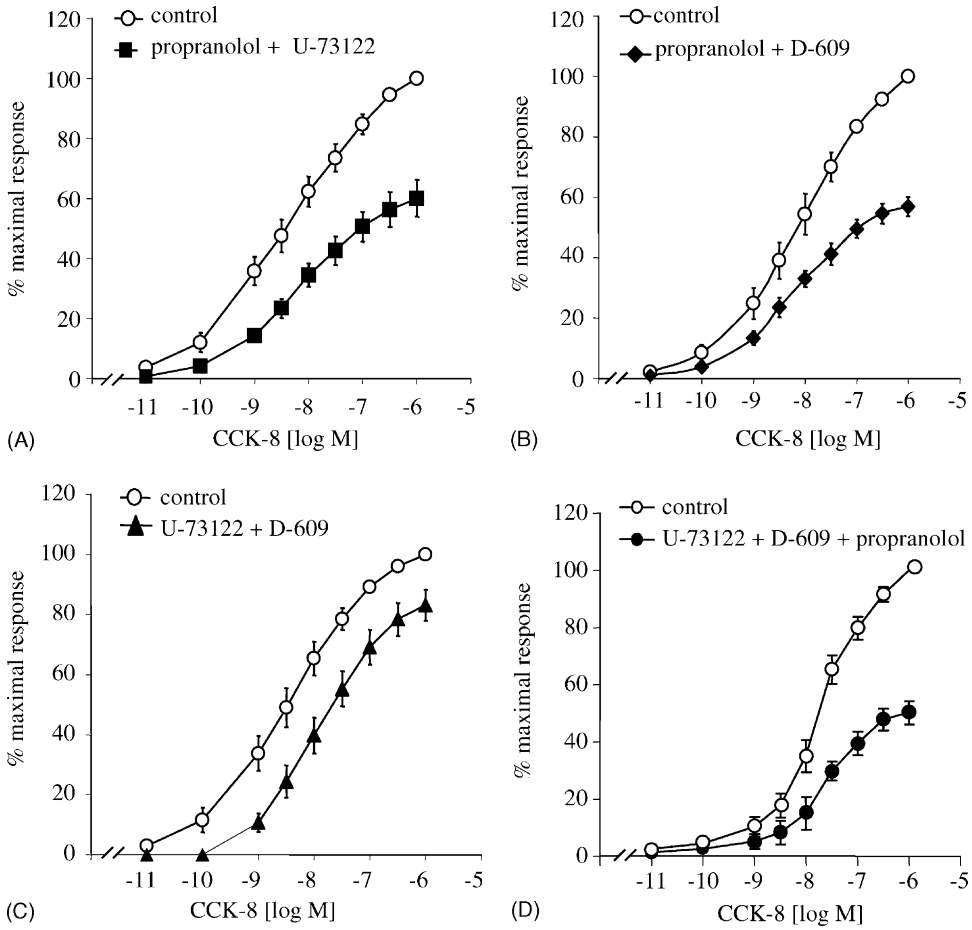

Fig. 4. Effect of the different combinations of the PL inhibitors on CCK-8-induced contraction. (A) Propranolol (100 μM) plus U-73122 (10 μM); (B) propranolol (100 μM) plus D-609 (100 μM); (C) U-73122 (10 μM) in combination with D-609 (100 μM); (D) propranolol (100 μM) plus U-73122 (10 μM) and D-609 (100 μM). Data points indicate means from 6 to 7 experiments and vertical lines show SE of the mean.

AA should induce contraction in gallbladder strips. Fig. 7 shows that AA induced a concentration-dependent contraction with an E_{max} of 15.4 ± 1.9 mN in response to 50 μM AA. The onset of tension development occurred within 41.0 ± 7.1 s for 3.2 μM AA, 58.0 ± 7.9 s in response to 10 μM AA, and 42.0 ± 4.2 s for 50 μM AA. The maximal tension was achieved after 3.05 ± 0.65, 5.29 ± 0.52 and 6.16 ± 0.45 min, at these concentrations of AA, respectively (N = 13).

The role of AA metabolites in the response to exogenous AA using indomethacin, NDGA or a mixture of both, to inhibit cycloxygenase and lipoxygenase was also investigated. Fig. 8A and C shows that indomethacin reduced by $71.2 \pm 13.3\%$ (N = 5, $P < 0.05$) and $75.5 \pm 11.7\%$ (N = 6, $P < 0.05$) the response to 10 and 50 μM AA, respectively. The presence of NDGA almost completely abolished the AA-induced contractions (94.5 ± 2.9 and $99.5 \pm 0.6\%$ of inhibition for 10 and 50 μM AA, respectively, N = 5, $P < 0.01$ for both concentrations; Fig. 8B and C), indicating that the contractile effect of AA is not direct but rather is mediated by AA metabolites. When added together, the two compounds did not induce further inhibition (data not shown).

It has been reported that CCK-induced gallbladder muscle contraction requires Ca^{2+} signals arising from influx of extracellular Ca^{2+} [23] and release of Ca^{2+} from intracellular stores [24,25]. Since it is assumed that $[Ca^{2+}]_i$ is the critical factor controlling the contractile machinery [4], if AA contributes to the CCK-induced contraction, the contractile effect of AA should show also be Ca^{2+}-dependent. Therefore, we tested the effects of the L-type Ca^{2+} channel blocker methoxyverapamil on AA-induced contractions. Methoxyverapamil (10 μM) significantly inhibited the contraction elicited by AA 50 μM ($41.3 \pm 8.1\%$ vs. $15.3 \pm 4.7\%$ normalized to ACh 10 μM response, N = 6, $P < 0.01$). This reduction was similar to that caused by the same concentration of methoxyverapamil in 320 nM CCK-8-induced contraction ($51.1 + 7.1\%$, N = 6, $P < 0.001$) and by replacing the normal K–HS with a Ca^{2+}-free solution immediately before CCK-8 application ($48.5 \pm 8.0\%$ of reduction for 320 nM CCK-8).

Another feature of the Ca^{2+} dependence of the CCK response is the sensitivity to CaM inhibitors, given that CaM mediates the effect of Ca^{2+} in smooth muscle contraction (see Fig. 2). As a putative contributor in the contractile effect of CCK, the AA-induced contraction

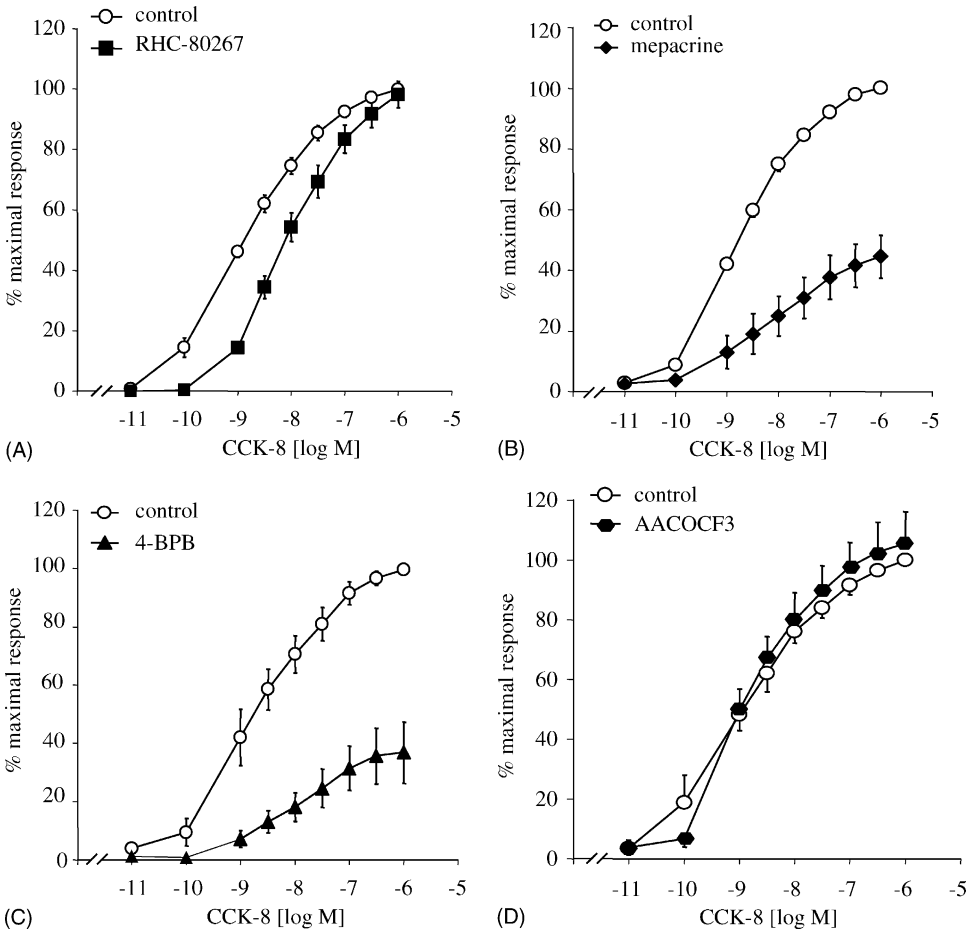

Fig. 5. Effect of the inhibitor of DAG lipase RHC-80267 (40 μM) (A) and the inhibitors of PLA_2 mepacrine (100 μM) (B), 4-BPB (100 μM) (C) and AACOCF3 (100 μM) (D) on CCK-8 concentration–response curve. Data points indicate means from 4 to 8 experiments and vertical lines show SE of the mean.

would be expected to be sensitive to CaM inhibition. When AA (10 and 50 μM) was added to the bath in the presence of the CaM inhibitor, W-7 (500 μM) no contractile response was detected ($N = 4$, $P < 0.001$ for each concentration).

4. Discussion

The overall aim of this study was to test the hypothesis that simultaneous activation of several PLs could contribute to CCK-8-induced gallbladder contraction. The results presented here indicate that activation of PI-PLC, PC-PLC and PLD participates in the contractile response to CCK-8, thus demonstrating a functional redundancy in the output of DAG that would reinforce the main role of this metabolite in the motor response to CCK-8. Moreover, we report here that the activation of PLA_2 may account for the role of AA in CCK-8-activated contraction and provide evidence of a link between DAG and AA output.

Smooth muscle contraction is ultimately triggered by phosphorylation of MLC_{20}. Phosphorylation of MLC_{20} involves the activation of MLC kinase by Ca^{2+}/CaM complex [3,26,27] and/or to inhibition of the MLC-phosphatase in response to DAG/PKC pathway [7]. Although PI-PLC is a ubiquitous enzyme which could account for the activation of these two messengers, our data show that CCK-8 also elicits contraction via production of DAG from multiple sources (see Fig. 9).

Upon G-protein coupled receptor stimulation, the relative contribution of the different PLs (PI-PLC, PC-PLC and PLD) to the production of the two second messengers, IP_3 and DAG, varies greatly between species and tissues [1,8]. For example, the activation of PI-PLC leads to IP_3-dependent Ca^{2+} mobilization in the circular layer of intestinal smooth muscle [28], while in the longitudinal layer, receptor-mediated elevation in $[Ca^{2+}]_i$ are due to Ca^{2+}-induced Ca^{2+} release (CICR), and this is triggered by Ca^{2+} influx in response to AA produced by PLA_2 [8]. Data presented here indicate that in gallbladder smooth muscle several PLs mediate the response to CCK-8 (see Fig. 9). Although selective inhibitors for PI-PLC and PC-PLC are not very effective when tested alone, a combination of both is clearly efficient to reduce the contraction. These results indicate that full activation of both enzymes is not necessary to attain a normal contraction in response to activation

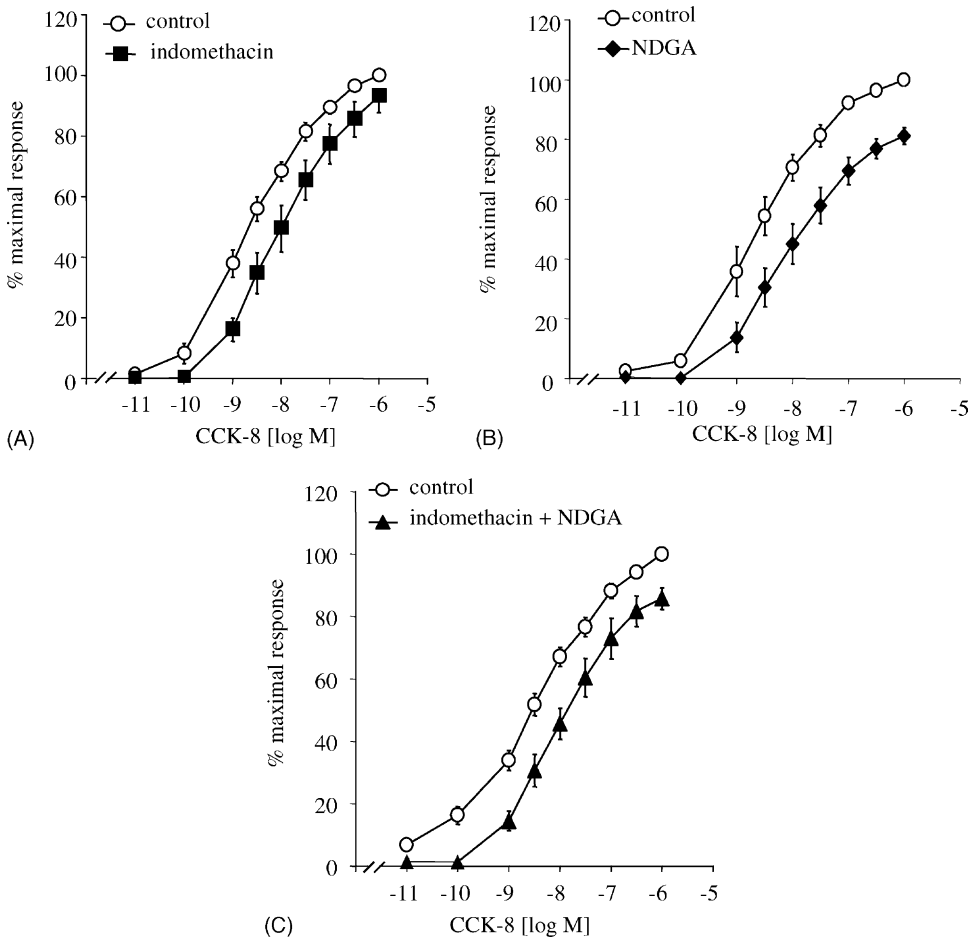

Fig. 6. Effects of the cycloxygenase inhibitor indomethacin (10 μM) (A), the lipooxygenase inhibitor NDGA (100 μM) (B) and the combination of both together (C) on the CCK-8-evoked contraction. Data points indicate means from 4 to 8 experiments and vertical lines show SE of the mean.

of the CCK receptor. The contribution of PLC seems to be more important for low levels of stimulation (see Fig. 3), emphasizing the putative importance of these enzymes in the physiological response of the gallbladder.

Besides PLC, PA-PH is also involved in the contraction, as shown by the effect of propranolol. Given that this enzyme catalyzes the breakdown of the PA generated by

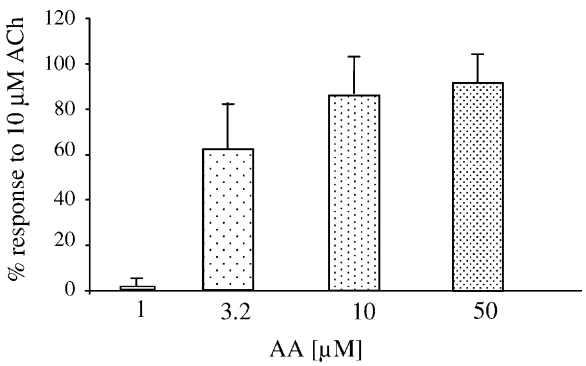

Fig. 7. Contractile gallbladder response to AA (1, 3.2, 10 and 50 μM AA) expressed as a percentage of the response to ACh 10 μM. Bars indicate means from 13 experiments and vertical lines show SE of the mean.

PLD from PC, this finding indicates that CCK also activates PLD (see Fig. 9). Although this is in contrast with the report of Yu *et al*. [1] showing that PLC, but not PLD, plays a role in the CCK action of cat gallbladder muscle, in vascular smooth muscle cells it has been found that activation of PLD and DAG metabolism comprise an important signaling cascade in angiotensin II-induced growth of this cells [29]. In addition, CCK also activates PLD in other cell types, such as pancreatic acinar cells [30]. Examination of the concentration–effect curves illustrates that the importance of this pathway increases with the level of stimulation, since propranolol clearly impairs contraction at presence of high levels of CCK-8. These results reveal the importance of DAG as a contractile messenger during CCK-8 stimulation. The activation of three pathways leading to a common product, DAG, could underlie the importance of this messenger in mediating the effects of the hormone. This also could be the sign of a functional redundancy to guarantee DAG production in situations where a concrete pathway is turn down.

From our results we can conclude that CCK-8 evokes gallbladder contraction via AA synthesis. Several lines of evidence support this conclusion: (1) inhibitors of

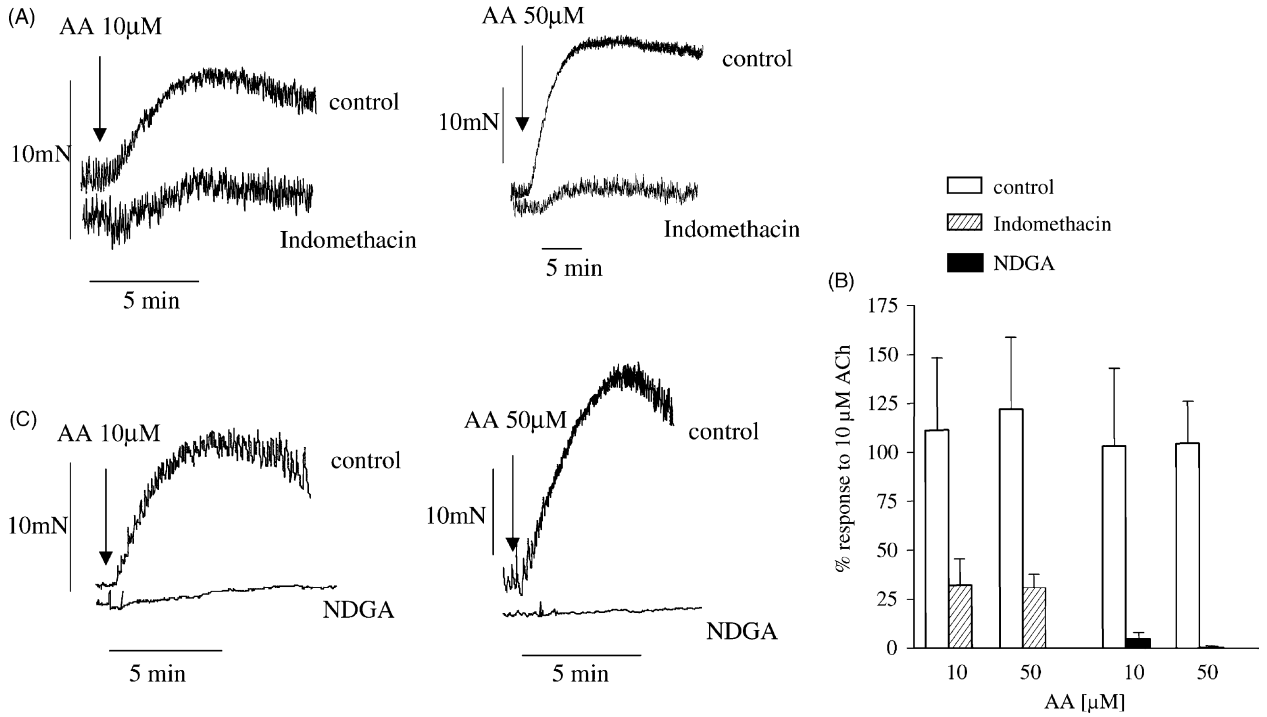

Fig. 8. (A and B) Original traces showing the response of the tissue to AA (10 and 50 μM) in absence (control) and in presence of indomethacin (10 μM, A) or NDGA (100 μM, B). The response to AA in presence of the inhibitors was tested after 7 hr to prevent the desensitization. (C) Summarized effects of indomethacin and NDGA on AA (10 and 50 μM)-induced contraction. Responses are expressed as percentage of the response to ACh 10 μM. Histograms indicate means ± SEM from five experiments.

AA-synthesizing enzymes decrease CCK-8-evoked contraction, (2) inhibitors of AA metabolism impair the contraction, (3) exogenous AA induces a contractile effect, and (4) the Ca^{2+} dependency shown by the response to exogenous AA is similar to the dependency shown by CCK-8.

The present data indicate that the sources of AA in stimulated gallbladder smooth muscle are both DAG lipase and PLA_2 (see Fig. 9). The former would act upon the DAG generated by PLs C and D, while the second enzyme cleaves AA directly from phospholipids [31,32]. The family of PLA_2 includes $cPLA_2$ and $sPLA_2$, based on kinetic, structural and localization criteria [31]. Although in most smooth muscle cells $cPLA_2$ seems to play a dominant role [15,33,34], in guinea pig gallbladder it is $sPLA_2$ that is involved in CCK-8-evoked contraction, given that mepacrine (a broad range inhibitor) and 4-BPB (an inhibitor specific for $sPLA_2$) had a strikingly similar effect in the curve of CCK-8, while AACOCF3, the inhibitor of $cPLA_2$, was without effect.

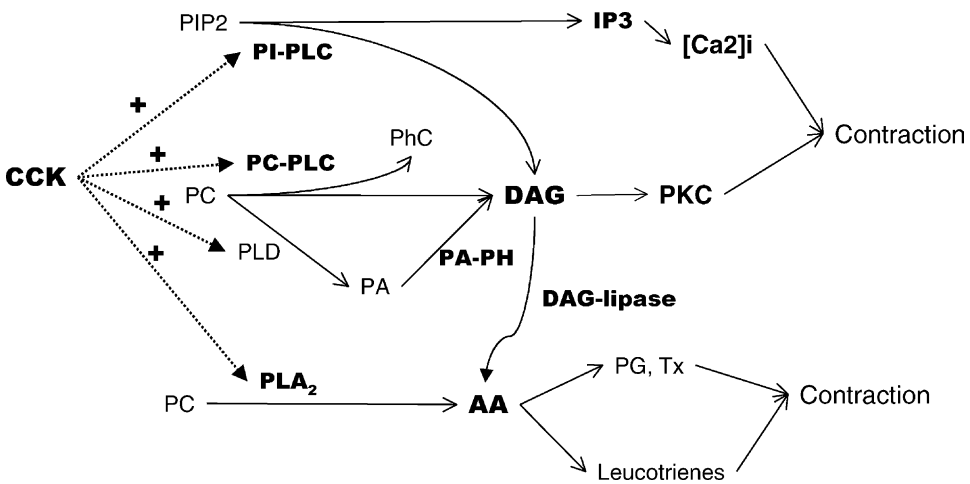

Fig. 9. Schematic diagram showing the intracellular pathways activated by CCK-8 to induce gallbladder contractions as concluded from our experimental evidences. The enzymes or metabolites studied are shown in bold.

The present finding that exogenous AA caused contraction in gallbladder is similar to previous reports in rat aorta [11]. However, while in aorta the effect of AA is direct, our data indicate that the contraction was due to cycloxygenase- and lypoxygenase-mediated metabolites, given the effects of indomethacin and NDGA on the contraction evoked by AA and by CCK-8. This is consistent with the participation of thromboxane A2 [12,13,35] and leukotrienes [15] in smooth muscle contraction.

Acute cholecystitis is characterized by (1) decreases in neurally-mediated contractions [36,37], (2) inhibition of the contraction induced by agonists that are membrane dependent such as CCK, ACh, KCl [36–38], (3) reduction in CCK-8 binding capacity [38], and (4) increases in PGE2 levels [38]. PGE2 has been shown to cause a concentration-dependent contraction [38,39] that remains unchanged in acute cholecystitis [38], which is consistent with a cytoprotective role for PGE2 in the gallbladder, at least in acute inflammation. The partial dependence of CCK-8-induced contraction on PG synthesis shown in our study, suggests that PGs may contribute to the remaining gallbladder contractile response to CCK in inflammation.

The fact that AA had the same dependency as CCK-8 with respect to extracellular Ca^{2+} entry and CaM activity (as revealed by the effects of methoxyverapamil and W-7) suggests that it is likely that AA metabolites act upstream of Ca^{2+} mobilization during stimulation with CCK-8. In fact, previous reports have shown that in smooth muscle AA modulates voltage-operated Ca^{2+} channels and sensitizes contraction via activation of MLCK [40–42]. Furthermore, in pancreatic acinar cells, it has been proposed that CCK mobilizes Ca^{2+} through AA [43].

Taken together, the results of the current study indicate that CCK-8-induced contraction of gallbladder smooth muscle involves the simultaneous activation of PI- and PC-PLC, PLD, PA-PH, DAG-lipase and PLA_2. This would produce, amongst other second messengers, DAG and AA, which would induce contraction through PKC activation in the case of DAG, and, in the case of AA, conversion into leukotrienes and prostaglandins (see Fig. 9). These autacoids participate in the contractile response upstream Ca^{2+}/CaM signal, probably modulating its activation and/or sensitizing the Ca^{2+} signaling mechanisms. However, the exact identity and mechanisms of action of AA metabolites requires further investigation.

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