Mechanism of cAMP-induced Ca^{2+} influx in *Dictyostelium*: role of phospholipase A_2

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cAMP-induced Ca^{2+} influx in *Dictyostelium* follows two pathways: a G-protein-dependent pathway where influx is reduced by 50–70 % in Gα2 and Gβ-negative strains and a heterotrimeric G-protein-independent pathway. Using a pharmacological approach, we found that phospholipase A_2 (PLA_2) is the target of both pathways. The products of PLA_2 activity, arachidonic acid (AA) and palmitic acid, induced Ca^{2+} influx to a similar extent as cAMP. Half-maximal activation occurred at 3 μM AA and saturation at 10 μM AA. The response to AA was quantitatively similar throughout early differentiation and thus independent of cAMP-receptor concentration. Synergy experiments revealed that cAMP and AA acted through identical pathways. The PLA_2-activating peptide, a peptide with sequence similarity to the G-protein β-subunit, activated Ca^{2+} influx. The G-protein-independent pathway was sensitive to genistein but not to blockers of protein kinase C and other kinases, suggesting that tyrosine kinase may directly or indirectly activate PLA_2 in this case.

INTRODUCTION

Signal transduction in *Dictyostelium* in response to cAMP binding to cell-surface receptors causes a transient increase of inositol trisphosphate and cGMP and an influx of Ca^{2+} (for reviews, see [1–4]). cAMP is subsequently synthesized and released and mediates chemotactic aggregation of the cells. The cytosolic Ca^{2+} increase is thought to regulate several Ca^{2+} binding proteins [5], motility and orientation [6], adenylyl cyclase activity [2,7] and calmodulin-binding proteins, e.g. calcineurin activity [8], as well as Ca^{2+} oscillations [3,9].

Ca^{2+} influx across the plasma membrane requires the activation of sarcoplasmic/endoplasmic-reticulum-type Ca^{2+}-ATPase(s) that transport Ca^{2+} into Ca^{2+} stores since the Ca^{2+} gradient across the plasma membrane is fairly small (1 μM extracellular versus 50–200 nM cytosolic). In addition, a V-type H^+-ATPase maintains an inside acidic pH to provide for Ca^{2+}/H^+ exchange [10–13].

The mechanism of Ca^{2+} influx is unknown. However, it seems to involve two different mechanisms: one is dependent on the Gα2-subunit of the G-protein and is thought to induce phospholipase C activation, whereas the other, being about of equal size, occurs independently of Gα2 [4,14]. A strong reduction in Ca^{2+} influx is also seen in the Gβ^- strain LW-14 [4,14]. Here we show that phospholipase A_2 (PLA_2) activity is required for most Ca^{2+} influx and that fatty acids can substitute for cAMP to elicit this response.

MATERIALS AND METHODS

Materials

Aristolochic acid [15], manoalide [16], 2-(*p*-amylcinnamoyl) amino-4-chlorobenzoic acid (ONO-RS-082) [17] and 1-palmitylthio-2-palmitoylamino-1,2-dideoxy-*sn*-glycero-3-phosphorylcholine [18], were from Biomol (Hamburg, Germany). *p*-Bromophenacyl bromide (BPB) [19] was from Sigma (Munich, Germany). Oleoyloxyethylphosphorylcholine [20], palmitoyl trifluoromethyl ketone [21] and (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one (halo enol lactone suicide substrate, HELSS) [22] were purchased from Calbiochem (Bad Soden, Germany), as well as the protein kinase C (PKC) inhibitors staurosporine, bisindolylmaleimide I and D-*erythro*-sphingosine. Isotetrandrine [23], genistein and RHC-80267 [1,6-bis(cyclohexyloximinocarbonyl amino)hexane] [24] were from Biomol, arachidonic acid (AA; sodium salt), 1,2-dioctanoyl-*sn*-glycerol (OOG) and 1-stearoyl-2-arachidonoyl-*sn*-glycerol (SAG) were from Fluka (Buchs, Switzerland). Phospholipase-A_2-activating protein (PLAP) was obtained from Bachem (Bubendorf, Switzerland).

Culture of cells

Wild-type strain Ax-2 was grown axenically as described previously [25]. Mutant strain JM-1 [26], which lacks the Gα2-subunit of the G-protein and constitutively overexpresses the cAMP receptor cAR1, was generously supplied by J. Milne (Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.). LW-14, a G-protein β-subunit null strain, overexpressing cAR1 [27], was from P. Devreotes (Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.). JM-1 and LW-14 were cultured axenically in Petri dishes. All the mutants were grown in the presence of 20 μg/ml geneticin (G418; Gibco–BRL, Paisley, Scotland).

For induction of development, exponentially growing cells were washed twice in ice-cold Sørensen phosphate buffer [17 mM $(KH_2/Na_2H)PO_4$ (pH 6.0)]. Cells (2×10^7/ml) were shaken until use at 23 °C.

Measurement of the extracellular Ca^{2+} concentration

Cells were washed in 5 mM Tricine/5 mM KCl (pH 7.0) and adjusted to a density of 5×10^7 cells/ml. Cell suspension (2 ml) was stirred and aerated in a cuvette and extracellular Ca^{2+} was determined with a Ca^{2+}-sensitive electrode (ETH 1001; Möller,

Abbreviations used: AA, arachidonic acid; PLA_2, phospholipase A_2; PLAP, PLA_2-activating protein; PKC, protein kinase C; BPB, *p*-bromophenacyl bromide; HELSS, *E*-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one; ONO-RS-082, 2-(*p*-amylcinnamoyl) amino-4-chlorobenzoic acid; DAG, diacylglycerol; SAG, 1-stearoyl-2-arachidonoyl-*sn*-glycerol; OOG, dioctanoyl-*sn*-glycerol; LPC, lysophosphorylcholine; CMF, conditioned medium factor.

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Zürich, Switzerland) as described previously [9]. The cell suspension buffers Ca^{2+}. Ca^{2+} scaling bars measure Ca^{2+} buffering capacity and were used to calculate absolute Ca^{2+} changes.

Ca^{2+} influx, determined by the electrode, reflects net Ca^{2+} influx, i.e. influx minus efflux. However, cAMP-stimulated Ca^{2+} influx, measured by $^{45}Ca^{2+}$, is of similar magnitude [28], indicating that efflux is not contributing much during the first 30–40 s, the time when influx is measured.

Basal, net Ca^{2+} influx was determined by addition of 1 μM $CaCl_2$ to the cell suspension. Influx was calculated by measurement of Ca^{2+} influx for 40 s.

RESULTS

Effect of PLA_2 inhibitors

We found that a variety of known effectors of PLA_2, at concentrations that inhibit PLA_2 activity in mammalian cells [15–23], inhibited cAMP-induced Ca^{2+} influx (Table 1). BPB, which does not act on the diacylglycerol (DAG)-lipase of neurons [24], blocked Ca^{2+} influx by more than 70 %. HELSS, a compound that generates a reactive bromoketone *in situ* and inhibits PLA_2 irreversibly [22], inhibited by approx. 60 %. Isotetrandrine, which interferes with G-protein coupling to PLA_2 but leaves enzyme activity intact [23], also blocked 57 % of the response. Manoalide, which is not absolutely specific for PLA_2 and was reported to inhibit phospholipase C and Ca^{2+} channels as well [29], inhibited 55 % of Ca^{2+} influx at 2 μM concentration. Another potent inhibitor was ONO-RS-082 [17], whereas other reported PLA_2 effectors were more or less inactive (Table 1).

In neurons, neurite outgrowth is triggered by nerve growth factor, which leads to AA release. In this case PLA_2 is not involved in signal transduction but DAG-lipase has been implicated [24]. We tested the inhibitor of DAG-lipase, RHC-80267, at 50 μM concentration and found no reduction of cAMP-stimulated Ca^{2+} influx (Table 1).

Table 1 Effect of inhibitors of PLA_2 and DAG-lipase on cAMP- and AA-induced Ca^{2+} influx in Ax-2 cells

Ca^{2+} influx was measured with a Ca^{2+}-sensitive electrode by addition of 1 μM cAMP or 6–10 μM AA to a suspension of 5×10^7 cells in the absence or presence of the drug at 4.5–8 h of starvation. Control influx amounted to 164 ± 29 pmol/10^7 cells for cAMP and 145 ± 29 pmol/10^7 cells for AA. Aristolochic acid was tested from 4 to 4.5 h. Control influx was 39 ± 7 pmol/10^7 cells. Addition of HELSS caused some impairment of the cells. Since AA-stimulated Ca^{2+} influx was unaffected by manoalide, we used the response to AA as a reference in this case. Cells were preincubated with the drugs for approx. 10 min. *n*, number of experiments is given in parentheses; n.d., not determined; OOPC, oleoyloxyethylphosphorylcholine; $PACOCF_3$, palmitoyl trifluoromethyl ketone; TPC, 1-palmitylthio-2-palmitoylamino-1,2-dideoxy-*sn*-glycero-3-phosphorylcholine.

Compound	Concentration (μM)	Inhibition of Ca^{2+} influx	
		cAMP (%)	AA (%)
HELSS	40–50	59 ± 10 (6)	0 (3)
Manoalide	2	55 ± 9 (4)	0 (2)
BPB	40	73 ± 21 (7)	n.d.
Isotetrandrine	40	57 ± 14 (6)	6.8 ± 10.7 (5)
ONO-RS-082	20–40	67 ± 25 (5)	n.d.
OOPC	10–25	20 ± 14 (3)	n.d.
$PACOCF_3$	30–50	19 ± 5 (4)	n.d.
TPC	40	0 (3)	n.d.
Aristolochic acid	50–100	0 (2)	n.d.
RHC 80267	50	0 (3)	0 (3)

Table 2 Effect on cAMP-induced Ca^{2+} influx by PLA_2-activating peptide (PLAP) or the DAG compounds SAG and OOG

Ca^{2+} influx was measured as described in Table 1, except for SAG and OOG, where cells were stimulated with 1 nM or 10 nM cAMP. *P* (significance relative to OOG) was calculated by using the Student's *t* test.

Addition	Activation (%)	Ca^{2+} influx of control (pmol/10^7 cells)	*n*	*P*
5 μM PLAP	51 ± 31	83 ± 32	3	0.006
50 μM SAG	53 ± 30	67 ± 31	8	0.0025
50 μM OOG	4 ± 6	74 ± 30	6	

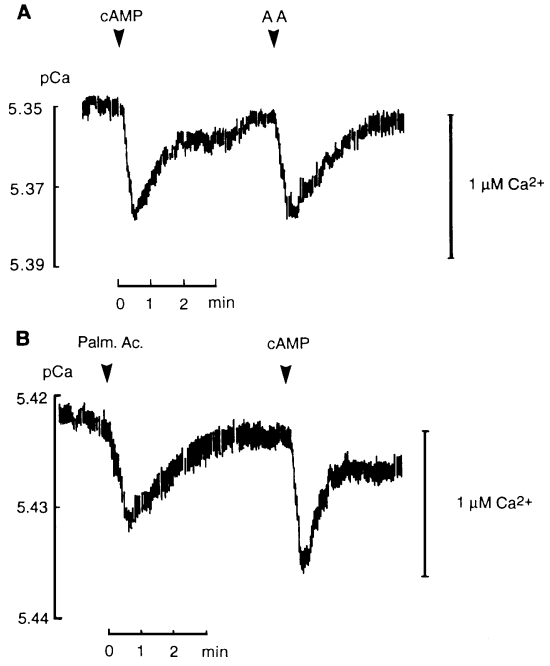

Figure 1 Ca^{2+} influx induced by cAMP and AA (A) or palmitic acid (Palm. Ac.) and cAMP (B)

The extracellular Ca^{2+} concentration of 5×10^7 Ax-2 cells per ml was recorded with a Ca^{2+}-sensitive electrode as described in the Materials and methods section. cAMP (1 μM) or the fatty acid (6 μM) were applied 6–7 h after induction of differentiation. One out of three separate experiments is shown.

PLA_2-activating agents

It has been shown previously that PLAP can activate AA release in intact pancreatic cells [30]. We found that a 5 μM concn. of the peptide increased cAMP-stimulated Ca^{2+} influx in each experiment with a mean of 50 % (Table 2). DAG is known to enhance PKC activity, as well as PLA_2 activity [31]. We analysed two different DAGs for activation. SAG at 50 μM concentration stimulated receptor-mediated Ca^{2+} influx in each experiment with a mean of 50 %, whereas OOG at the same concentration was without effect (Table 2), indicating that PLA_2 is stimulated but not PKC, since PKC would have been activated by both compounds. This conclusion is supported by the finding that the PKC inhibitors staurosporine (1 μM), bisindolylmaleimide (10–20 μM) and D-*erythro*-sphingosine (40 μM) did not affect Ca^{2+} influx to a significant extent (results not shown), excluding a prominent role for PKC in regulating cAMP-dependent Ca^{2+} influx.

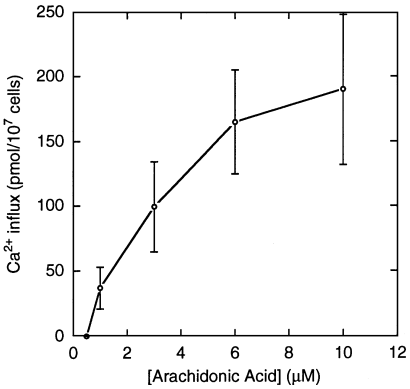

Figure 2 Dose–response curve for AA-induced Ca^{2+} influx in Ax-2 cells

See legend of Figure 1 for details. The number of experiments were: 2, 4, 16, 45, 6 (0.5–10 μM AA). The measurements were performed 4–8 h after induction of differentiation.

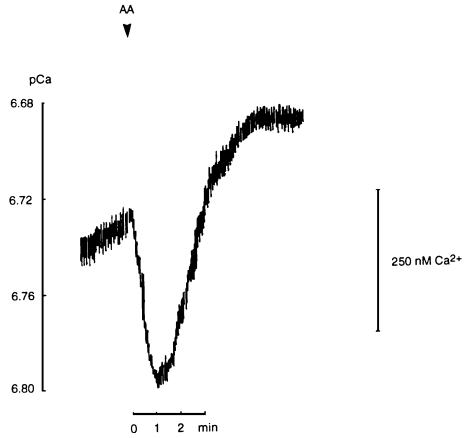

Figure 3 AA-induced Ca^{2+} influx at low external Ca^{2+} concentrations

After induction of differentiation (5 h) 10 μM AA was applied to 5×10^7 Ax-2 cells per ml in the presence of 200 nM extracellular Ca^{2+}. One out of eight independent experiments is shown.

Fatty acids induce Ca^{2+} influx

If cAMP-stimulated PLA_2 activity was required for Ca^{2+} influx, then either product, lysophosphorylcholine (LPC) or fatty acids, should be active. Indeed fatty acids were active: Figure 1 shows that AA, as well as palmitic acid, induced a rapid, transient reduction in the extracellular Ca^{2+} concentration, whereas LPC was inactive (results not shown). This transient behaviour is characteristic of chemotactic responses in *Dictyostelium*, since endogenous activation occurs in a pulsatile manner [1,3]. Ca^{2+} influx across the plasma membrane is thought to occur through as-yet-unidentified Ca^{2+} channels and efflux is thought to occur by Ca^{2+}-ATPase activity. Both differ in their sensitivity to azide, the latter being more sensitive [32].

A dose–response curve demonstrates the potent activity of AA, which elicited a half-maximal effect at an approx. 3 μM concentration (Figure 2). Ca^{2+} influx occurred at a low external Ca^{2+} concentration of 200 nM. Under these conditions, the Ca^{2+} gradient is very small (200 nM versus 50 nM inside) and substantial influx can only occur if Ca^{2+} transport into the stores is activated (Figure 3). AA acted downstream of PLA_2 activity,

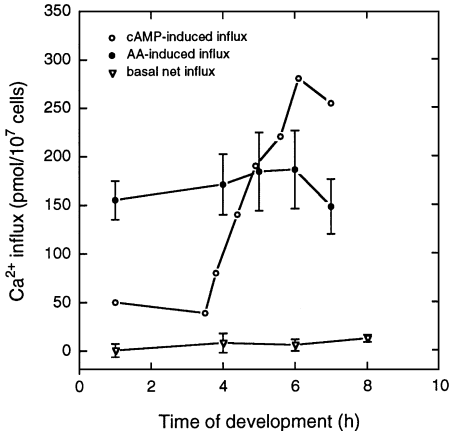

Figure 4 Ca^{2+} influx during differentiation to aggregation competence

Ax-2 cells were assayed following addition of 6 μM AA or 1 μM $CaCl_2$ for basal net influx (see the Materials and methods section) to the cell suspension. In comparison, cAMP-induced Ca^{2+} influx is shown (results taken from [14]). (○) cAMP-induced influx; (●) AA-induced influx; (▽) basal net influx. Values ± S.D. of at least four separate determinations are presented.

since neither HELSS, manoalide nor isotetrandrine inhibited the AA-induced Ca^{2+} influx (Table 1).

AA was active throughout early development until aggregation (Figure 4), in contrast to cAMP, which exhibits only low activity early on in differentiation and then peaks during aggregation [14,28,33].

Do cAMP and AA act synergistically?

To test whether both compounds stimulate Ca^{2+} influx through independent or identical pathways, synergy experiments were performed, in which cells were first treated with a pulse of cAMP or AA. Then, 20 to 25 s later, as soon as the Ca^{2+} influx reached its maximum, a second pulse of the other compound was administered to the cells, while continuously monitoring the cellular response. As shown in Figure 5, addition of a saturating dose of cAMP, followed by a saturating dose of AA, induced only a small additional influx. The same was true if AA was given first or when both compounds were added simultaneously. This result shows that both compounds share an identical pathway to elicit Ca^{2+} influx. By contrast, as shown previously, PAF (1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) and cAMP displayed synergistic effects on Ca^{2+} influx [14].

PLA_2 is activated by two separate mechanisms

To assess the mechanism of PLA_2 activation, we analysed two mutant strains deficient in heterotrimeric G-proteins, JM-1 and LW-14, that lack a functional Gα2-subunit and the only known β-subunit respectively, but overexpress the cAMP receptor cAR1 required for full responsiveness of the cells [4]. As shown previously [14,26], LW-14 displayed only a small amount of cAMP-induced Ca^{2+} influx. The response to 10 μM AA, although reduced with respect to Ax-2, was much larger and increased further at 60 μM AA (Table 3). In JM-1, Ca^{2+} influx in response to 10 μM AA was smaller than for cAMP. However, increasing the stimulus to 60 μM AA resulted in a large Ca^{2+} influx (Table

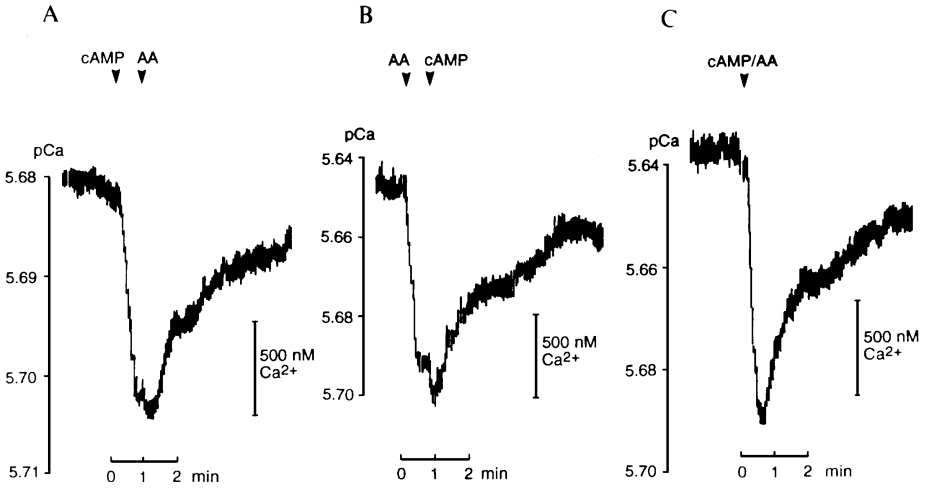

Figure 5 Stimulation of Ca^{2+} influx by cAMP and AA added sequentially

cAMP (10 μM) was added to a cell suspension at 5–6 h of development and Ca^{2+} influx was monitored. At the maximum of influx, 10 μM AA was given to elicit a further response, as shown by arrowheads in (**A**). In (**B**) AA was added first, followed by cAMP, whereas in (**C**) both compounds were applied simultaneously. (**A**) 207 + 18 pmol/10^7 cells; (**B**) 212 + 36 pmol/10^7 cells; (**C**) 259 pmol/10^7 cells. This experiment was performed three times with essentially the same result.

Table 3 AA-induced Ca^{2+} influx in the β^- strain, LW-14, and the Gα2$^-$ strain, JM-1, in relation to Ax-2

For comparison cAMP-induced Ca^{2+} fluxes are shown. The mutants were allowed to develop for 4–6 h, as was done with Ax-2 cells. JM-1 cells do not develop at all; LW-14 cells do not aggregate. Measurements were performed as described in the Materials and methods section. Values are Ca^{2+} influx in pmol/10^7 cells ± S.D. The responses to both 10 μM and 60 μM AA in the mutant strains are different from the cAMP responses at $P < 0.0001$, as determined by using the Student's *t* test. *n*, number of experiments; n.d., not determined.

Addition	Ax-2	LW-14	JM-1
1 μM cAMP	202 ± 90 ($n = 44$)	38 ± 23 ($n = 36$)	84 ± 27 ($n = 40$)
10 μM AA	190 ± 58 ($n = 5$)	88 ± 36 ($n = 11$)	21 ± 15 ($n = 4$)
60 μM AA	n.d.	121 ± 23 ($n = 7$)	157 ± 39 ($n = 4$)

Table 4 Inhibition of cAMP-induced Ca^{2+} influx in JM-1 cells

cAMP (1 μM) was added to a suspension of 5×10^7 cells/ml in the absence or presence of the drug at 1–5 h of starvation and Ca^{2+} influx was determined with a Ca^{2+}-sensitive electrode. Ca^{2+} influx in control cells amounted to 203, 124, 183, 84 and 66 pmol/10^7 cells.

Compound	Concentration (μM)	Inhibition (%) (*n*)
HELSS	40	72 ± 10 (3)
Isotetrandrine	40	0 (3)
BPB	40	81 ± 18 (3)
Genistein	140	78 ± 22 (2)
	300	85 ± 18 (4)

3). Apparently, the sensitivity of JM-1 and LW-14 to AA is reduced compared with Ax-2 cells. In these strains, AA by-passes G-protein-linked PLA_2 activation, leading to a larger Ca^{2+} influx compared with cAMP. These results thus show that a large part of receptor-activated Ca^{2+} influx is mediated by heterotrimeric G-proteins that act upstream of PLA_2 activity.

Does the remaining G-protein-independent Ca^{2+} influx in strain JM-1 also depend on PLA_2 activity? This question was addressed using the PLA_2 inhibitors HELSS and BPB. Table 4 shows that both components inhibited cAMP-induced Ca^{2+} influx to a large extent. Isotetrandrine was inactive, as expected, since no Gα2-protein-mediated activation of PLA_2 is possible in this strain. Moreover, this result renders the interaction of PLA_2 with other Gα-proteins at this stage of differentiation unlikely.

An alternative mechanism for activation of PLA_2 is phosphorylation by PKC or tyrosine kinases [34]. Above we have shown that PKC was not involved in the regulation of Ca^{2+} influx. Incubation of Ax-2 cells with inhibitors of protein kinase A (5 μM KT5823, $n = 2$), protein kinase G (1–4 μM KT5720, $n = 4$) and calmodulin kinase II (10–30 μM KN62, $n = 5$) did not alter cAMP-induced Ca^{2+} influx. However, influx was inhibited by the specific tyrosine-kinase blocker genistein [35] by 51 ± 15 % (100–300 μM, $n = 14$). In JM-1 cells inhibition by genistein increased to 80 % (Table 4), suggesting that most of the Ca^{2+} influx in strain JM-1 is regulated by tyrosine phosphorylation and not by heterotrimeric G-proteins, whereas in Ax-2 cells both mechanisms are operating.

To address the question of whether genistein acted upstream or downstream of PLA_2 activity, we tested the AA-induced Ca^{2+} influx in the absence or presence of genistein. We found only marginal inhibition of the AA response by genistein in Ax-2 cells (8 ± 8 % for 6 μM AA versus 63 ± 6 % for 1 μM cAMP, $n = 4$), indicating that the regulation by tyrosine kinase activity occurs upstream of PLA_2.

DISCUSSION

We found that PLA_2 activity mediates a large part of the receptor-stimulated Ca^{2+} influx in *Dictyostelium*. This conclusion is based on the following findings. (i) Five out of nine different PLA_2 inhibitors blocked the response to 50–70 % (Table 1). Note

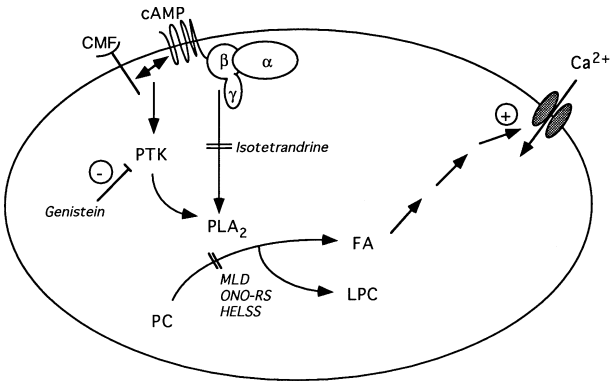

Figure 6 Hypothetical scheme for receptor-mediated Ca^{2+} entry in *Dictyostelium* with respect to PLA_2 activation

Two separate pathways lead to stimulation of PLA_2: (i) via a heterotrimeric G-protein inhibited by isotetrandrine and (ii) directly or indirectly by tyrosine phosphorylation, which is inhibited by genistein. The latter pathway is proposed to operate in the Gα2 mutant JM-1. Fatty acids, but not LPC, are able to induce Ca^{2+} entry. MLD, manoalide; FA, fatty acid; PTK, protein tyrosine kinase.

that the inhibition by isotetrandrine cannot amount to 100% because approx. 50% of Ca^{2+} influx is G-protein independent. (ii) PLAP, as well as the DAG compound SAG activated Ca^{2+} influx (Table 2). (iii) Non-esterified fatty acids (AA and palmitic acid) could substitute for cAMP to induce Ca^{2+} influx. (iv) AA acted on an identical pathway with cAMP (Figure 5). (v) AA seemed not to be generated by DAG-lipase activity.

It is important to note that the half-maximal activity of AA was found to be 3 μM, thereby excluding non-physiological reactions of AA reported to occur at higher concentrations. Although it has been published that *cis*-unsaturated fatty acids at 50–200 μM concentration inhibit binding of cAMP to its receptor [36], we found no impairment of cAMP-induced Ca^{2+} influx by AA at the low concentrations employed. In 26 experiments, the cAMP response amounted to 100 ± 36%, 6–10 min after the addition of 6 μM AA. Moreover, the action of AA seemed to be independent of cAMP receptors since, in contrast to cAMP, the amount of Ca^{2+} influx remained unchanged during differentiation to aggregation competence when cAMP receptors reach peak levels. The fatty acid reacted downstream of PLA_2 activity, because HELSS, manoalide and isotetrandrine did not reduce Ca^{2+} influx induced by AA. Palmitic acid could substitute for AA, indicating that it is the activity of AA itself that is required and not its oxidation products.

Several mechanisms exist to regulate PLA_2 activity in mammalian cells. PLA_2 was reported to be activated by the β-subunit of G-proteins [30]. Moreover, the PLA_2-activating peptide, PLAP, exhibits sequence similarities to the β-subunit. The following results are in agreement with the possibility that the β-subunit activates PLA_2 in *Dictyostelium* as well: (i) a 70% inhibition of cAMP-induced Ca^{2+} influx was found for the β null strain LW-14, (ii) AA had a much higher activity in LW-14 compared with cAMP (Table 3), (iii) a 50% activation of the cAMP response occurred in the presence of PLAP (Table 2).

In addition, our results concerning the inhibition of PLA_2 activity in JM-1 cells (Table 4) show that there must be a G-protein-independent pathway for activation of PLA_2 as well. It has been reported in mammalian cells that PKC and tyrosine kinase are regulators of PLA_2 [34]. Inhibition of PKC in *Dictyostelium* did not lead to a significant alteration in Ca^{2+} influx; however, strong inhibition occurred in the presence of genistein, a specific tyrosine-kinase blocker (Table 4). Genistein acted upstream of PLA_2, since the response to AA was not changed in its presence. Tyrosine kinases are known in *Dictyostelium*, and one major substrate is actin. Tyrosine phosphorylation of actin was inhibited to approx. 50% by 80 μM genistein [37]. We found approx. 80% inhibition of Ca^{2+} influx in JM-1 cells with 140 μM genistein, suggesting that PLA_2 is activated directly or indirectly by a tyrosine kinase. Since the cAMP receptor requires co-activation by conditioned medium factor (CMF), a conditioned medium factor of 80 kDa, which binds to a separate receptor [38], it is conceivable that an additional signal-transduction pathway is activated by CMF in conjunction with cAMP.

In Figure 6 we present a hypothetical scheme for signal transduction of the receptor-mediated Ca^{2+} influx with respect to PLA_2. cAMP-binding in conjunction with CMF to cell-surface receptors activates PLA_2 through the transient stimulation of a heterotrimeric G-protein. The interaction of the G-protein with PLA_2 is blocked by isotetrandrine. An alternative pathway for PLA_2 activation is inhibited by genistein, suggesting that a protein tyrosine kinase mediates this effect. This pathway is proposed to be operating in the Gα2 mutant JM-1. PLA_2 hydrolyses phosphorylcholine to yield LPC and a long-chain fatty acid. Only the latter induced Ca^{2+} influx across the plasma membrane, whereas several inhibitors of PLA_2 activity like manoalide, HELSS or ONO-RS-082 blocked Ca^{2+} influx.

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