

The role of calcium ions in the process of acetyltransferase activation during the formation of platelet-activating factor (PAF-acether)

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The role of Ca^{2+} in the activation of the enzyme lyso-(platelet-activating factor):acetyl-CoA acetyltransferase was studied in rat peritoneal macrophages in response to complement-coated zymosan particles and ionophore A23187. By using Ca^{2+} -containing buffers, a threshold concentration of extracellular Ca^{2+} above $1\ \mu\text{M}$ was found to be necessary to observe the activation of the enzyme in response to zymosan. By contrast, a significant role of intracellular Ca^{2+} in this process could be ruled out, since the putative intracellular calcium-transport antagonist TMB-8 [8-(*NN*-diethylamino)octyl-3,4,5-trimethoxybenzoate] did not inhibit the activation of the acetyltransferase induced by zymosan in the presence of extracellular Ca^{2+} . The link between acetyltransferase activation and extracellular Ca^{2+} transport was studied by measuring Ca^{2+} uptake in response to the stimuli. Zymosan particles induced a rapid increment in cell-associated Ca^{2+} which correlated well with the extent of acetyltransferase activation ($r = 0.91$) and with the release of platelet-activating factor ($r = 0.95$) in response to different doses of zymosan. Cellular Ca^{2+} efflux in response to zymosan particles was also measured and found to be increased, as compared with controls, when the activation of the acetyltransferase declined. In short, the data suggest that the entry of extracellular Ca^{2+} into the cell is a crucial event in the activation of acetyltransferase and, thereby, in the formation of platelet-activating factor in rat peritoneal macrophages.

Variations in the concentration of free intracellular Ca^{2+} have been implicated in the mechanisms of stimulus–secretion coupling in a number of cell types that participate in inflammatory reactions (Gallin & Rosenthal, 1974; Goldstein *et al.*, 1974; Freedman *et al.*, 1975; Ishizaka *et al.*, 1980). These variations begin either after a ligand–receptor interaction, which favours an early increment in cellular Ca^{2+} , or by complexing Ca^{2+} with an ionophore, which allows free movement of the complex across the cell. In addition, some secretory responses are more dependent on Ca^{2+} from intracellular sources than on extracellular Ca^{2+} (Mustard & Packham, 1970).

Abbreviations used: Hepes; 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; PAF-acether, platelet-activating factor.

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The secretion of PAF-acether has been studied in a number of cellular systems, and recent evidence suggests that the formation of this mediator is dependent on a step which involves the transfer of an acetyl moiety from acetyl-CoA into lyso-PAF-acether. In previous work from this laboratory, the activation of the enzyme lyso-PAF-acether:acetyl-CoA acetyltransferase in human polymorphonuclear leucocytes after phagocytosis of complement-coated zymosan particles has been described (Alonso *et al.*, 1982a). These findings have been confirmed by other authors (Albert & Snyder, 1983; Ninio *et al.*, 1983) in rat macrophages, but thus far there is no information on the mechanisms involved in the activation of the acetyltransferase and, therefore, on the mechanisms which regulate the final step in the formation of PAF-acether.

In the present paper we describe our findings on the role of Ca^{2+} , both extracellular and intracellular in origin, in the process of activation of the

enzyme acetyltransferase, which occurs during the stimulation of rat peritoneal macrophages in response to complement-coated zymosan particles and to the Ca^{2+} ionophore A23187.

Materials and methods

Chemicals and reagents

Lyso-PAF-acether was from Bachem Feinchemikaliem, Bubendorf, Switzerland. [^3H]acetyl-CoA (1.3 Ci/mmol) was from New England Nuclear Corp. $^{45}\text{CaCl}_2$ (12.5 mCi/mg) was from Amersham International. TMB-8 [8-(*NN*-diethylamino)octyl-3,4,5-trimethoxybenzoate] was generously given by Dr. Viggo Esmann, Department of Medicine, Marselisborg Hospital, Aarhus, Denmark. Ionophore A23187 was from Boehringer. Zymosan was from Sigma.

Purification of macrophages

Macrophages were obtained from the peritoneum of Sprague-Dawley rats by the method of Bloom *et al.* (1967). Cells were collected in a HEPES-buffered medium containing (g/litre): HEPES, 4.77; KCl, 0.37; NaCl, 8.18; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1; glucose, 1; bovine serum albumin, 2.5. The cells were washed twice by centrifugation in the same medium and resuspended in the same medium supplemented with 1 mM- CaCl_2 . Purified macrophages were obtained by plating cells in plastic dishes and removal of non-adherent cells. Adherent cells were detached by adding cold HEPES-buffered medium containing 1 mM-EDTA and incubating at 4°C for 15 min. Adherent cells were then gently dislodged by rinsing with a Pasteur pipette, washed twice in HEPES-buffered medium and resuspended in Ca^{2+} -supplemented buffer. Cell viability was assessed by Trypan Blue exclusion and found to be greater than 95%. From the outcome of control experiments in peritoneal cells non-adhered to plastic dishes, we could rule out any significant effect of the isolation procedure on Ca^{2+} transport.

PAF-acether released by stimulated cells was assayed and characterized as described previously (Alonso *et al.*, 1982b). The release of PAF-acether is expressed as the percentage of radioactivity released by rabbit platelets prelabelled with [^3H]5-hydroxytryptamine in response to 100 μl of supernatant.

Assay of acetyltransferase activity

Peritoneal macrophages were incubated at 37°C at a density of 10^7 cells/ml and stimulated with zymosan particles coated with complement or by ionophore. At various times after stimulation,

samples containing 5×10^6 cells were centrifuged 5 min at 400g at 4°C in precooled tubes and resuspended in 0.1 M-Tris/HCl, pH 7.4. After a 15 s sonication in a Branson B-12 sonifier equipped with a micro-tip, at position 3, the homogenate was used for the enzymic biosynthesis of PAF-acether as described previously (Wyckle *et al.*, 1980; Alonso *et al.*, 1982a). The homogenate was incubated at 37°C for 10 min in 1 ml of medium containing 0.1 M-Tris/HCl, pH 7.4, 10 μg of lyso-PAF-acether and 0.5 μCi of [^3H]acetyl-CoA. The reaction was stopped by adding chloroform/methanol (1:2, v/v), and phase formation was achieved by adding chloroform and water. The organic phase was then removed, dried under N_2 , and the labelled lipids quantified after separation by t.l.c. with propionic acid/propanol/chloroform/water (2:2:1:1, by vol.). This procedure permits the separation of PAF-acether from lyso-PAF-acether, phosphatidylcholine, lysophosphatidylcholine and neutral lipids.

Uptake of radiolabelled Ca^{2+}

$^{45}\text{Ca}^{2+}$ uptake was measured by a slight modification of the procedure of Naccache *et al.* (1977). Suspensions of macrophages were incubated with $^{45}\text{Ca}^{2+}$ (5 $\mu\text{Ci}/\text{ml}$) plus 1 mM unlabelled Ca^{2+} for 40 min at 37°C . The cells were then challenged with complement-coated zymosan particles or the Ca^{2+} ionophore A23187, and, at the indicated times, 0.3 ml aliquots were layered on top of Eppendorf microcentrifuge tubes containing 100 μl of silicone oil. The separation of the cells from the suspending medium was accomplished by a single centrifugation (45 s at 6000g). The pelleted cells were isolated by cutting off the bottom of the tubes and placing these tube bottoms in scintillation vials containing 1 ml of Triton X-100 in 5% (v/v) HNO_3 . The cell pellets were then dislodged from the tube tips by forceful aspirations with Pasteur pipettes and vigorous mixing. Radioactivity was determined by scintillation spectrometry after addition of an appropriate scintillation cocktail.

Measurement of ^{45}Ca efflux

For efflux experiments the cells were preincubated for 40 min at 37°C . They were then centrifuged for 2 min at 1000g at 4°C , and the cell pellet was transferred to 1.5 ml Eppendorf tubes containing no radioactivity for three new washes. At the end of this period, the cells were resuspended in the desired volume of thermally equilibrated buffer and divided into the number of tubes needed. Radioactivity was assessed in supernatants and pellets by liquid-scintillation spectrometry.

Results

Effect of extracellular and intracellular Ca²⁺ on the activation of the acetyltransferase

We examined the threshold of free Ca²⁺ (Ca_f²⁺) required for induction of the fraction of acetyltransferase activation dependent on extracellular Ca²⁺. For this purpose we used a series of Hepes-buffered media containing EGTA (1mM) and various concentrations of CaCl₂ to obtain a range of final Ca_f²⁺ values between 1 μM and 10mM. Ca_f²⁺ was calculated on the basis of an affinity constant of Ca²⁺ for EGTA of 4.4 × 10⁵ M⁻¹ (Ogawa, 1968). As shown in Fig. 1, the process of activation of the acetyltransferase by zymosan particles was dependent on extracellular Ca²⁺, with a Ca_f²⁺ threshold above 1 μM. Maximal enzyme activation was observed with 10mM-Ca_f²⁺.

The role of intracellular Ca²⁺ was studied by using TMB-8, a local anaesthetic which modulates cellular responses by a mechanism which most likely involves immobilization of intracellular calcium stores (Malagodi & Chiou, 1974; Chiou & Malagodi, 1975). The experiments were carried out in the presence and absence of extracellular Ca²⁺

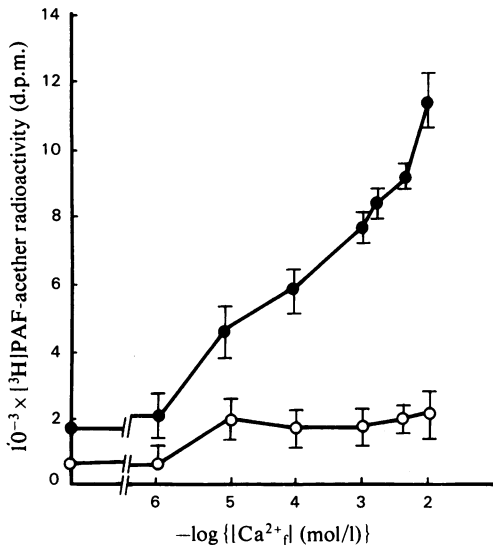


Fig. 1. *Effect of extracellular Ca²⁺ on the activation of acetyltransferase by zymosan particles*

Cells were stimulated with complement-coated zymosan particles (●) at a density of 2mg/10⁷ cells in the presence of different concentrations of extracellular free Ca²⁺. At 5 min after zymosan addition, samples containing 5 × 10⁶ cells were taken and used for acetyltransferase assay. Controls included cell samples in the absence of phagocytosable zymosan particles (○). Results are means ± s.d. for five experiments performed in duplicate.

either in resting cells or after stimulation by complement-coated zymosan particles. As shown in Fig. 2, optimal enzyme activation required extracellular Ca²⁺. TMB-8 induced a significant increment in enzyme activation as compared with that observed in the absence of this compound. This result was considered surprising, and therefore new experiments to measure Ca²⁺ fluxes in the presence of TMB-8 were performed.

Effect of complement-coated zymosan particles and ionophore A23187 on acetyltransferase activity and cell-associated Ca²⁺

The addition of phagocytic particles to rat macrophages induced a rapid and significant increase in cell-associated ⁴⁵Ca²⁺. This occurred as early as 30s after the addition of particles and

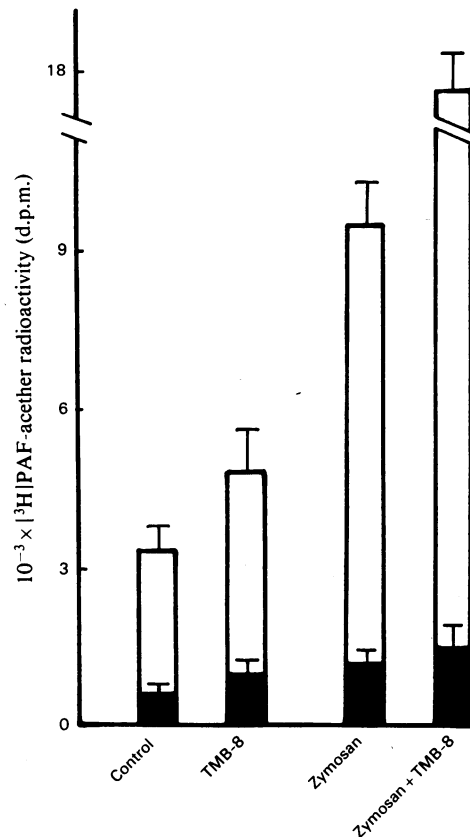


Fig. 2. *Effect of TMB-8 on acetyltransferase activation*

Peritoneal macrophages were stimulated with zymosan particles in the presence or absence of Ca²⁺, and TMB-8 (100 μM). Acetyltransferase activity was assayed in cell pellets 5 min after the addition of zymosan particles. Results are means ± s.d. for three experiments performed in duplicate. □, 1mM-Ca²⁺; ■, no Ca²⁺, 1mM-EGTA.

remained for some time. In order to rule out the effect of non-specific $^{45}\text{Ca}^{2+}$ trapping in the extracellular space or within phagolysosomes, appropriate controls with [^{14}C]inulin showed that this trapping represented less than 0.5% of the total radioactivity. $^{45}\text{Ca}^{2+}$ associated with zymosan particles was measured by including appropriate controls of zymosan particles without cells. As shown in Fig. 3, $^{45}\text{Ca}^{2+}$ uptake preceded acetyltransferase activation during the initial minutes. When ionophore A23187 was used as a stimulant, $^{45}\text{Ca}^{2+}$ uptake preceded acetyltransferase activation as well. In order to assess whether the increment in cell-associated Ca^{2+} represents a net increase in total cell Ca^{2+} or only an increase in the exchangeability of some pool of Ca^{2+} without a net gain by the cell, Ca^{2+} efflux was measured. As shown in Fig. 4, Ca^{2+} efflux in phagocytosing macrophages remained parallel with that observed

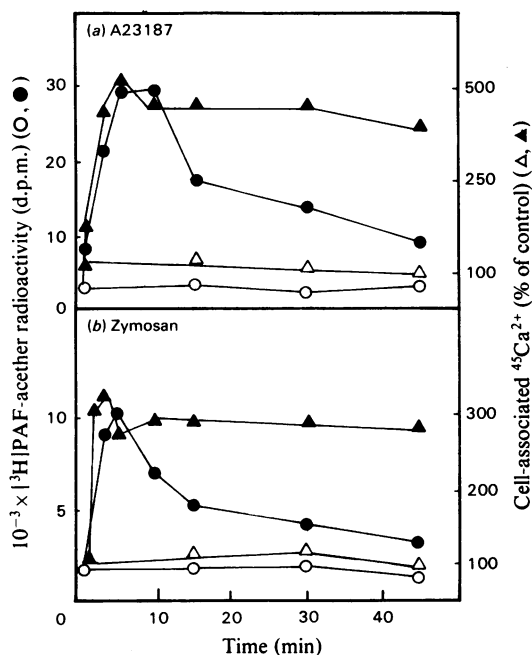


Fig. 3. Effect of ionophore A23187 (a) and zymosan particles (b) on the time-course of acetyltransferase activation and cell-associated Ca^{2+}

Cells were incubated at a density of 10^7 cells/ml with $^{45}\text{Ca}^{2+}$ for 40 min. At the end of this period, $5 \mu\text{g}$ of ionophore or 2 mg of zymosan particles were added. At the indicated times, samples were taken for the assay of acetyltransferase activity and cell-associated Ca^{2+} . Open symbols represent control cells; closed symbols represent stimulated cells. Cell-associated Ca^{2+} is expressed as a percentage of that of control cells.

in resting cells during the initial 5 min; thereafter, a net increase in Ca^{2+} efflux was observed in response to zymosan. Cell-associated Ca^{2+} correlated well with PAF-acether release ($r = 0.95$) and acetyltransferase activation ($r = 0.91$), as shown in Fig. 5.

In order to assess whether the action of TMB-8 on acetyltransferase is related to an action of the

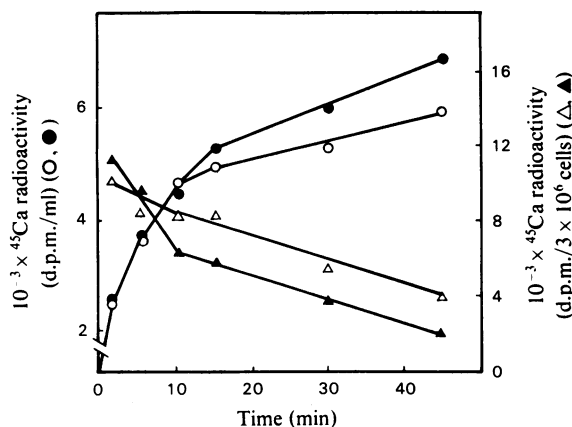


Fig. 4. Effect of zymosan particles on calcium efflux. Peritoneal macrophages were labelled with ^{45}Ca and then washed to remove extracellular Ca^{2+} . At the times indicated, cells were pelleted and radioactivity measured in cells (Δ, \blacktriangle) and supernatants (O, \bullet). Open symbols represent control cells; closed symbols represent phagocytosing macrophages. Results are mean values for a typical experiment performed in duplicate.

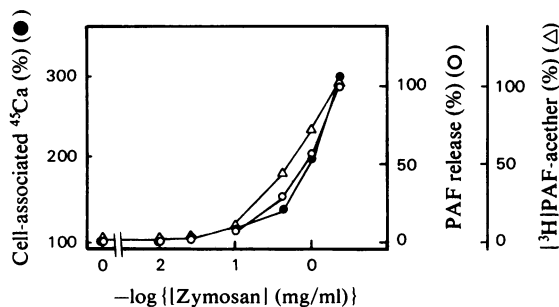


Fig. 5. Correlation between the extent of acetyltransferase activation, PAF-acether release, cell-associated Ca^{2+} and the concentration of zymosan particles

Cells were taken 5 min after the addition of zymosan to assess acetyltransferase activity (Δ), PAF-acether release (O) and cell-associated ^{45}Ca (\bullet). Results are means for a typical experiment performed in duplicate and are expressed as percentages of values found in the absence of zymosan particles.

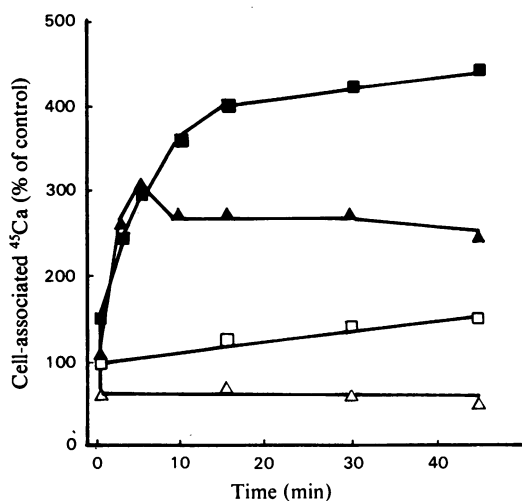


Fig. 6. Effect of TMB-8 on cell-associated Ca²⁺ in response to ionophore

Cells were incubated with ⁴⁵Ca for 40 min in the presence (□, ■) or absence (△, ▲) of 100 μM-TMB-8. At the end of this period, ionophore (5 μg/ml) was added and samples taken at the times indicated. Open symbols represent control cells; closed symbols represent stimulated cells. Results are averages for two experiments performed in duplicate.

compound on Ca²⁺ transport, cell-associated ⁴⁵Ca²⁺ was measured in the presence of this drug. As shown in Fig. 6, preincubation with TMB-8 induced a net increase in cell-associated ⁴⁵Ca²⁺ both in resting conditions or in response to ionophore.

Discussion

The results presented here confirm previous findings on the activation of the enzyme 1-alkyl-2-lysoglycerophosphocholine:acetyl-CoA acetyltransferase in rat macrophages (Albert & Snyder, 1983; Ninio *et al.*, 1983) and human polymorphonuclear leucocytes (Alonso *et al.*, 1982a) in response to various stimuli. This seems of interest because this enzyme catalyses the last step in the formation of PAF-acether, an inflammatory mediator with a wide spread of biological actions (Benveniste *et al.*, 1972; Demopoulos *et al.*, 1979; Goetzl *et al.*, 1980; Sánchez Crespo *et al.*, 1982; Humphrey *et al.*, 1982). In a previous paper (Sánchez Crespo *et al.*, 1983) we have proposed that the regulation of the acetyltransferase represents a crucial event in the generation and release of PAF-acether, since the agents which elicit PAF-acether formation in human polymorphonuclear leucocytes activate the enzyme; by contrast, stimulants that induce a cell response, but do not

release PAF-acether, fail to do so. Furthermore, the acetyltransferase reaction seems to be inhibited in resting cells.

Another limiting step in the formation of PAF-acether could be the formation of acetyl-CoA by an acetyl-CoA synthase; however, this step does not depend on Ca²⁺ entry, since Chap *et al.* (1981) have demonstrated that incorporation of [³H]-acetate in neutral lipids occurs in both resting and ionophore-stimulated cells; by contrast, only ionophore-stimulated cells showed the label to migrate as PAF-acether.

In previous papers we have shown that cyclic nucleotides and phospholipid methylation could influence the release of PAF-acether from human polymorphonuclear leucocytes in response to zymosan particles (García Gil *et al.*, 1981; Alonso *et al.*, 1982b); for this reason we first attempted to find an association between intracellular levels of cyclic nucleotides or the degree of phospholipid methylation on the one hand and the extent of acetyltransferase activation on the other. However, our findings were negative, and we decided to study the role of Ca²⁺. Our present data show that activation of acetyltransferase depends on the availability of extracellular Ca²⁺ at concentrations as low as 1–10 μM. By contrast, intracellular Ca²⁺ does not seem to be involved in this process, because the activation is not observed in the absence of extracellular Ca²⁺. Furthermore, the compound TMB-8, an antagonist of intracellular Ca²⁺ transport (Malagodi & Chiou, 1974; Chiou & Malagodi, 1975) not only failed to block the activation of the enzyme, but increased the activation of the enzyme induced by stimulants in the presence of extracellular Ca²⁺.

The link between extracellular Ca²⁺ and acetyltransferase activation was further studied by measuring radiolabelled-Ca²⁺ uptake and Ca²⁺ efflux. Our data show that a net increase in cell-associated Ca²⁺ precedes the activation of the enzyme, and cell-associated Ca²⁺ correlates well with the extent of acetyltransferase activation. In addition the blockade of intracellular Ca²⁺ transport induces an increase in both cell-associated Ca²⁺ and acetyltransferase activation. This paradoxical effect of the inhibition of intracellular Ca²⁺ transport has been observed by other authors (Shaw & Lyons, 1982) studying the different Ca²⁺ pools involved in the activation of rabbit platelets in response to thrombin and PAF-acether. In fact, Shaw & Lyons (1982) reported that, whereas TMB-8 blocked the response of rabbit platelets to PAF-acether, the addition of TMB-8 induced an increment in the [³H]5-hydroxytryptamine released in response to thrombin and ionophore A23187, compounds whose actions are entirely dependent on the availability of extracellular Ca²⁺. Our data

agree with those of Shaw & Lyons (1982) in showing the enhancing effect of intracellular Ca^{2+} -transport blockade on processes dependent on extracellular Ca^{2+} . Furthermore, our data suggest that this effect could be explained by the blockade of the entry of Ca^{2+} into internal pools not involved in the process of cellular activation.

The existence of separate Ca^{2+} pools in macrophages can be sustained by a number of observations. Firstly, the existence of mitochondria in macrophages explains the existence of a respiration-dependent mitochondrial membrane potential and a Ca^{2+} flux. Secondly, macrophages display membrane potentials which are the result of an increase in the K^+ conductance of the membrane as a result of a rise in intracellular Ca^{2+} (Gallin *et al.*, 1975). Thirdly, localization of Ca^{2+} occurs on the cytoplasmic side of the plasma membrane during chemotaxis (Cramer & Gallin, 1979). However, we do not know which are the events intermediate between external- Ca^{2+} entry and increased PAF-acether production, that is, the role of calmodulin and phosphorylation reactions.

The mobilization of intracellular Ca^{2+} in many systems depends on the production of natural ionophores which promote the release from stores. Thus lipoxygenated products of arachidonic acid have been reported to increase significantly the uptake of Ca^{2+} by polymorphonuclear leucocytes (Naccache *et al.*, 1981), and pharmacological blockade of the lipoxygenase pathway abolishes Ca^{2+} uptake by platelets in response to PAF-acether (Lee *et al.*, 1983). It is unlikely that TMB-8 could block these ionophoretic mechanisms in our system, because it does not block enzyme activation.

Although our data strongly suggest a role for Ca^{2+} in the activation of the acetyltransferase, we do not know which are the ultimate steps mediating between Ca^{2+} entry and enzyme activation.

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