of hyperpolarizing current pulses (not shown). Thus, although the accumulation of intracellular Ca^{2+} can produce an increase in resting potassium conductance (*26*), the results reported here indicate that at least one major component of potassium conductance, activated during depolarization, becomes depressed rather than activated by Ca^{2+} accumulation.

There are several possible means by which the calcium-dependent depression might occur. The simplest suggestion is that activation of $I_{K(Ca)}$ leaves it in a fatigued condition. This hypothesis seems unlikely, however, since the maximum depression takes time to develop (Fig. 2C) and since the effect is well developed after pulse I depolarizations too small to evoke a substantial outward current (Fig. 1A). The delayed depressing action of Ca^{2+} on $g_{K(Ca)}$ might also be a secondary consequence of a calcium-mediated change in intracellular *p*H. This is also unlikely because the currents and their depression remained unchanged in the presence of a CO_2-carbonate buffer, *p*H 7.1, reported to lower the intracellular *p*H of snail neurons (*15*). Another possibility is that internal calcium receptors [analogous to the acetylcholine receptors of the end-plate postsynaptic membrane (*27*)] desensitize on prolonged exposure to Ca^{2+}. The inward calcium current might initially activate $I_{K(Ca)}$, and accumulated intracellular Ca^{2+} might bind to a secondary site so as to interfere with potassium current. In any event, the calcium-dependent late depression of outward current cannot be due to a depression in Ca^{2+} influx and hence a reduced activation of $I_{K(Ca)}$, for Ca^{2+} entry has been shown to facilitate rather than depress with repetitive depolarization (*8, 28, 29*). Undoubtedly, such facilitation of a partial inward current contributes to the observed depression of net outward current. However, a true decrease in outward K^+ movement was previously shown to be correlated with depression of the pulse II net outward current (*6*).

Physiological correlates of the delayed depression of the outward current by intracellular Ca^{2+} accumulation can be seen in a train of action potentials (Fig. 2D). The increase in overshoot, the slowing of the repolarizaiton, and the positive shift in undershoot which occur during successive impulses are all consistent with a progressive reduction in activation of outward current. This reduction may be a correlate of the increment in intracellular Ca^{2+} resulting from Ca^{2+} influx known to occur during each action potential (*30, 31*). Prolongation of the action potential has been reported to occur in presynaptic branches with repetitive firing (*32*). Spike prolongation, due in part to a depression of the potassium system, may contribute to increased entry of Ca^{2+} and thus play a role in presynaptic facilitation.

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16. Depression of pulse II current is generally maximal at potentials corresponting to the rising portion of the pulse I *I-V* curve (that is, somewhat more negative than the peak of the calcium-dependent component of the *I-V* plot). A similar negative displacement along the voltage axis is seen in the peak aequorin signal relative to the peak of the calcium-dependent component of the *I-V* plot when the calcium-sensitive photoprotein is used to monitor free Ca^{2+} levels simultaneously with membrane currents (R. Eckert and D. Tillotson, unpublished results). These similarities lend further support to the interpretation that the pulse II outward current depression is related to pulse I Ca^{2+} entry.
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Inhibition of a Lymphocyte Membrane Enzyme by Δ^9-Tetrahydrocannabinol in vitro

Abstract. *Delta-9-tetrahydrocannabinol (Δ^9-THC) inhibited the activity of lysolecithin acyl transferase, a membrane-bound lymphocyte enzyme, at concentrations above 1.3 μM. Stimulation of acyl transferase activity by concanavalin A, an early response in lymphocyte activation, was entirely abolished in the presence of Δ^9-THC.*

Controversy exists as to the effect of Δ^9-tetrahydrocannabinol (Δ^9-THC) on the immune system. It has been reported that lymphocyte activation in vitro can be inhibited by Δ^9-THC concentrations as low as 1.6 μM (*1*) although this has been disputed (*2*). The lipophilic nature of Δ^9-THC suggests that any such action would be mediated at the level of the lymphocyte plasma membrane, and it has been shown that the early acceleration of phospholipid turnover during lymphocyte transformation can be blocked by Δ^9-THC (*3*). Other workers have suggested that later intracellular events in the transformation process, such as DNA synthesis, are the site of Δ^9-THC inhibition (*4*). It is clear from other studies (*5*) that perturbations of membrane structure by lipophilic substances may have a profound influence on intracellular events in lymphocyte transformation. We now describe the inhibition by low levels of Δ^9-THC of a membrane-bound lymphocyte enzyme that normally participates in the events of transformation. Lysolecithin acyl transferase (E.C. 2.3.1.23) catalyzes the formation of lecithin from lysolecithin and coenzyme A–activated fatty acids. As such, the enzyme has an important role in maintaining or altering membrane structure. Its level in T (thymus-dependent) lymphocytes is rapidly increased by mitogens such as concanavalin A (Con A) (*6*). We report here the complete

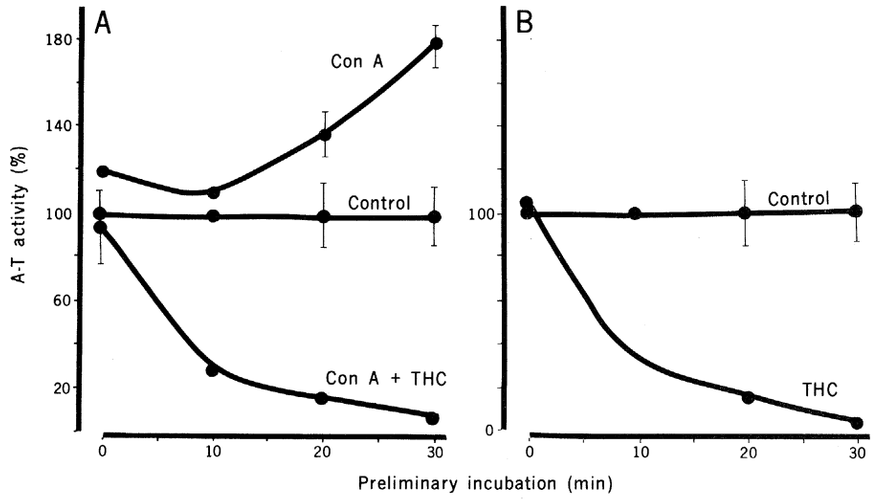

Fig. 1. Effect of Δ^9-THC on acyl transferase activity in the presence (A) and absence (B) of Con A. One milliliter of RPMI 1640 containing 10^7 lymphocytes was incubated at 37°C with 10 μg of Con A, 10 μM Δ^9-THC in 50 μl of dimethyl sulfoxide or Con A and Δ^9-THC for various times as described in the text. Controls were first incubated with 50 μl of dimethyl sulfoxide only. Acyl transferase activity was assayed by incubating the cells for 10 minutes at 37°C with 25 nmole of oleoyl-CoA and 15 to 30 nmole of [^{32}P]lysolecithin after the preliminary incubation period. The amount of ^{32}P radioactivity incorporated into lecithin in controls represents 100 percent activity. All assays were performed in duplicate, and the standard deviation is shown. The differences were significant ($P < .025$) by Student's t-test for Con A stimulation at the preliminary incubation times of 20 and 30 minutes, and for Δ^9-THC inhibition at 10, 20, and 30 minutes.

inhibition of basal and Con A–stimulated acyl transferase activity in the presence of Δ^9-THC concentrations above 1.3 μM.

Lymphocytes were prepared by gently homogenizing spleens of mice in Hanks balanced salt solution with a Teflon-glass homogenizer. Red blood cells were removed (*7*), the resultant white cells were adjusted to 10^7 cell/ml in RPMI 1640 medium, and their viability was checked by the trypan blue exclusion test. Portions (1 ml) of cells were incubated at 37°C for 10, 20, or 30 minutes or not at all (controls) in the presence of either Con A (10 μg/ml) or 10 μM Δ^9-THC in 50 μl of dimethyl sulfoxide (or both) before the assay for enzyme activity. Similar lymphocyte preparations were incubated for 30 minutes in the presence of various concentrations of Δ^9-THC in dimethyl sulfoxide before assay. Dimethyl sulfoxide had no effect on enzyme activity at the concentrations used. Acyl transferase activity was assayed by adding 25 nmole of oleoyl–coenzyme A (oleoyl-CoA) and 15 to 30 nmole of ^{32}P-labeled lysolecithin (*8*) in 0.1 ml of H_2O directly to the lymphocyte preparation. The mixture was incubated for a further 10 minutes at 37°C. Lipids were extracted in a chloroform, methanol, water system (1 : 1 : 0.8) and separated by thin-layer chromatography (*9*). The extent of acylation was calculated by comparing radioactivity (counts per minute) detected in the lecithin band with that in the lysolecithin band. Controls contained only 50 μl of dimethyl sulfoxide and were normalized to 100 percent acyl transferase activity, with all other results being calculated relative to this value. Viability studies based on the trypan blue exclusion test indicated that no significant effect on the survival of the lymphocytes occurred during incubation.

Prior incubation for 30 minutes with Con A gave maximal stimulation of the enzyme, and significant stimulation could be detected at a 20-minute incubation (Fig. 1A). Considerable inhibition of activity by 10 μM Δ^9-THC occurred in the presence of Con A (7.6 percent activity) or in the absence of Con A (5.5 percent activity). In the absence of Con A, Δ^9-THC gave complete inhibition of activity at 1.3 μM and half-maximal inhibition (K_i) at 0.35 μM (Fig. 2). No inhibition could be detected below 0.1 μM. The 2000-fold excess of substrate concentration over the Δ^9-THC concentration makes it unlikely that the inhibition is a result of lowered substrate concentration due to interaction of Δ^9-THC and substrate.

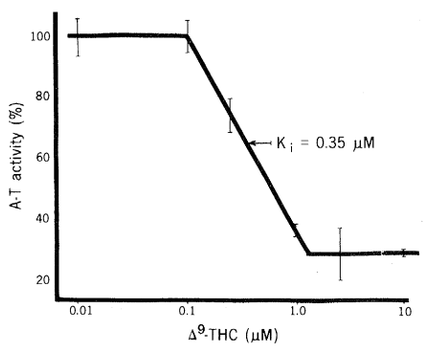

Fig. 2. Effect of Δ^9-THC on acyl transferase activity. Portions (1 ml) of RPMI 1640 containing 10^7 lymphocytes were incubated at 37°C for 30 minutes with Δ^9-THC in 50 μl of dimethyl sulfoxide. Acyl transferase activity was assayed thereafter by incubating the cells for 10 minutes at 37°C with 25 nmoles oleoyl–CoA and 15 to 30 nmoles [^{32}P]lysolecithin. The amount of ^{32}P incorporated into lecithin in controls that contained no Δ^9-THC represents 100 percent activity. All assays were run in duplicate, and the mean ± standard deviation is shown.

The earliest detected biochemical events in lymphocyte transformation are those involving lipid turnover, especially fatty acid metabolism (*10*). These changes precede the other changes, such as increased DNA and RNA synthesis by 16 hours or more (*11*). One of the causes of increased phospholipid fatty acid turnover induced in lymphocytes by mitogens such as Con A, is the increased acyl transferase activity (*6*). This increase in enzyme activity can be abolished by low concentrations of Δ^9-THC, and this effect may be responsible for an observed inhibition of fatty acid incorporation into phospholipids by 10 μM Δ^9-THC (*12*). It is of interest that substances that increase the fluidity of membranes, such as decanol, chlorpromazine, 4-chlorophenol, and barbital (*13*) will inhibit acyl transferase activity if present at much higher concentrations than that of Δ^9-THC, namely greater than $10^{-4} M$. This suggests that low concentrations of Δ^9-THC can induce changes in the lipid phase of the lymphocyte membrane that are inhibitory to the membrane-bound enzyme.

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Temporal Control of Urate Oxidase Activity in *Drosophila*: Evidence of an Autonomous Timer in Malpighian Tubules

Abstract. *The appearance of urate oxidase activity in the Malpighian tubules of* Drosophila melanogaster *is synchronized with the time of emergence of the imago from the puparium. A developmental clock within the Malpighian tubules specifies the time of appearance of urate oxidase activity.*

Different cell types exhibit their repertoire of proteins in a definite sequence and at a characteristic time during development. A description of the events which control the time of appearance and disappearance of tissue- and stage-specific proteins is of fundamental importance for an understanding of the mechanisms giving rise to the differentiated state.

At least two types of phenomena may be involved in the temporal control of a tissue-specific protein. The phenotype of a cell may be modified by signals emitted from closely apposed or noncontiguous cells at a fixed time during development (*1*). Conversely, an intracellular clocklike mechanism may specify the time of occurrence of a change in the differentiated state. Clocklike phenomena during development have been referred to in discussions of programmed cell death (*2, 3*), aging (*3, 4*), the differentiation of distal wing structures from the progress zone (*5*), and the appearance of an enzyme in mouse trophoblast cells in vitro (*6*).

Mechanisms have been proposed by which a cell might autonomously control the timing of developmental events. These include an intracellular counter for the number of DNA replications or cell divisions (*3, 4, 7*), an accumulation of substances to a threshold level (*8*), or a causally connected progression of intracellular reactions which subsequently activate transcription of a specific genetic locus (*9*). In addition to the above, Bonner (*10*) has speculated about the possible role of free-running endogenous oscillators in the timing of nonperiodic developmental events.

This investigation of the mechanism specifying the time of appearance of urate oxidase activity in the Malpighian tubules of *Drosophila melanogaster* was initiated to evaluate experimentally the role of autonomous biological clocks and humoral factors in the temporal control of a discrete tissue-specific biochemical event. Urate oxidase (E.C. 1.7.3.3) catalyzes the conversion of uric acid to allantoin and, in *Drosophila*, is located exclusively in the Malpighian tubules (*11*), a tissue that does not undergo cell division or reorganization after the larval stage. Urate oxidase activity is not detectable during embryonic development, in first and second instar larvae, or in pupae, but is present in third instar larvae and adults (*11, 12*). Our data indicate that the appearance of urate oxidase activity in the tubules upon emergence of the adult from the puparium is controlled by a developmental clock within the Malpighian tubules (*13*).

The appearance of urate oxidase activity upon emergence is most easily detected when there is a large burst of activity. Therefore, in all experiments we employed a xanthine dehydrogenase–deficient strain (*14*) which has five- to tenfold more urate oxidase activity than wild-type *Drosophila* when measured 1 day after emergence (*11*).

The *Drosophila* were raised at 25°C in constant light on cornmeal, molasses, and yeast food (*15*). Constant light was used because this condition extinguishes the rhythmic pattern of emergence (*16*) which then occurs without delay when development of the imago is complete.

The time remaining until emergence of an adult from the puparium is determined by selecting pupae with yellow, orange, and light brown eyes. These pupae are heterogeneous with respect to the time remaining until emergence which ranges from 37 to 50 hours at 25°C (*17*). Ten hours after the pupae with yellow, orange, and light brown eyes are collected, pupae with red eyes and very light gray wings are selected at hourly intervals. This second step assures that the pupae are viable and synchronized at the same developmental stage. The light gray wing stage lasts approximately 1 hour. Ninety-eight percent of xanthine dehydrogenase–deficient pupae with red eyes and gray wings emerge as adults after 26.5 ± 3 hours when incubated at 25°C.

Malpighian tubules were dissected in *Drosophila* Ringer solution, *p*H 6.9 (*18*), gently aspirated into a glass transplant needle, and inserted into the abdomen of a host. The transplant procedure for *Drosophila* tissues is described by Ursprung (*19*). A pair of anterior tubules and a pair of posterior tubules each connected to their respective collecting ducts were separately transplanted into different hosts anesthetized with ether. After transplantation the hosts were maintained on petri dishes (20 by 100 cm) containing a moistened Kimwipe and a dish (2.2 by 0.7 cm) containing 5 percent dextrose and 1 percent agar. This procedure allowed for easy retrieval of a host after the petri dish was cooled. For the first few days after emergence the presence or absence of food does not influence the level of urate oxidase activity in xanthine dehydrogenase–deficient strains (*11*).

To retrieve the host tubules and transplanted donor tubules, the hosts were submerged and dissected in 0.25*M* sucrose containing 1.7 m*M* EDTA, *p*H 6.9, at 22° to 25°C. After the abdomen was opened, the donor tubules usually floated free and could always be distinguished from the host's tubules which were attached to the alimentary canal. A pair of posterior and anterior donor tubules and one complete set of host tubules were then separately assayed for urate oxidase activity.

Modifications of a radiochemical assay for urate oxidase previously reported (*20*) were used for the detection of activity in the Malpighian tubules from a single *Drosophila*. One complete set of tubules was rapidly transferred on the tip of a dissecting needle to a 1.5-ml Brinkmann test tube. The assay tubes contained 11.0 m*M* boric acid, 1.4 m*M* EDTA (disodium salt), and 0.18 percent Triton X-100 in a total volume of 15 μl at *p*H 9.05. The tubule was incubated in the assay mixture for 20 minutes at 0° to 4°C. This process disrupted the cells and released urate oxidase into the incubation medium. At the completion of the incubation period, 10 μl of 0.59 m*M* (10 mg/100 ml) [2-^{14}C]uric acid (50 to 53 mc/mmole; Amersham/Searle) was added to initiate the reaction carried out at 26°C. After 2 minutes and again at 6 minutes, a 5-μl sample of the reaction mixture was removed and spotted on one of ten sites which were 2.0 cm apart and 1.5