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Inhibition of cyclic AMP efflux by insect pheromones and fatty acids

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Avian erythrocytes export cyclic AMP by a means that prostaglandins A_1 and A_2, but not other eicosanoids, inhibit ($EC_{50} \approx 45$ nM). Several insect pheromones and the fatty acyl components of common membrane phospholipids also inhibit cyclic AMP efflux ($EC_{50} \approx 30$ μM). The presence of at least one double bond in the acyl chain enhances the effect. Unlike PGA, fatty acids probably do not act via formation of a glutathione adduct but very likely by altering membrane fluidity. Inhibition of cyclic AMP export provides a mechanism by which products of phospholipid metabolism can influence the cyclic AMP signaling pathway.

cyclic AMP; Transport; Fatty acid; Pheromone; Membrane phospholipid

1. INTRODUCTION

Cyclic AMP escapes from all bacterial, acrasial, and metazoal cells in which this aspect of cyclic AMP metabolism has been examined [1]. In the pigeon erythrocyte, cyclic AMP export occurs by a process that has many characteristics of active transport, is distinct from the synthesis and degradation of cyclic AMP [2], and may be distinguished pharmacologically from the transporters of amino acids, glucose, adenosine, K^+ (both the Na^+,K^+-ATPase and furosemide-inhibitable, isoproterenol-stimulatable NaCl-KCl symport), and SO_4^{2-} (band III) [3]. Prostaglandins of the A series (PGA_1 or PGA_2) inhibit cyclic AMP efflux from avian red cells, putatively via their intracellular conversion to glutathione adducts at carbon 11 [4,5].

In trying to probe further the inhibition of cyclic AMP transport from pigeon red cells we have tested molecules bearing some structural similarities to PGA_1. We find that certain fatty acids (including common constituents of membrane phospholipids) as well as certain insect pheromones also inhibit the extrusion of cyclic AMP from these cells.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

trans-10-Dodecene-(1,9)-diol was synthesized by Grignard addition of 1-bromo-8-tetrahydropyranyloxyoctane to crotonaldehyde, followed by de-protection under mild acidic conditions and purification of the diol product by silica gel 'flash' chromatography. Other compounds tested were purchased from Sigma (St. Louis, MO). RO20-1724 was a generous gift from Hoffman La Roche, Inc. (Nutley, NJ).

2.2. *Procedures*

Blood was drawn from the wing vein of a pigeon (*Indion mondian*) into a heparinized syringe, diluted 25-fold into cold isotonic saline containing 10 mM EDTA, and centrifuged at 250 × *g* for 5 min. The buffy coat was removed and the red cells were resuspended and centrifuged twice more in Earle's salts (containing, in g/l: NaCl, 6.8; KCl, 0.4; $NaH_2PO_4 \cdot H_2O$, 0.14; $MgSO_4 \cdot 7H_2O$, 0.2; $NaHCO_3$, 2.2) with 2 g/l glucose, equilibrated with 95% O_2/5% CO_2. After two more cycles of centrifugation the cells were suspended at 10% hematocrit in Earle's solution containing the phosphodiesterase inhibitor RO20-1724 at 50 μM.

To measure extrusion of cyclic AMP separately from its synthesis, cells were incubated with isoproterenol (10 μM) for 10 min at 30°C diluted 25-fold into cold Earle's solution, centrifuged at 250 × *g* for 5 min, and resuspended into fresh

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Earle's solution (containing 50 μM RO20-1724). These cells were then divided into aliquots and placed into tubes containing putative inhibitors of transport, placed at 37°C, and aliquots were removed at timed intervals into a microfuge tube containing a drop of inert phthalate ester and centrifuged for 30 s in a Beckman Microfuge B to separate the cells from the medium containing extruded cyclic AMP. Trichloroacetic acid (10% final) and a tracer amount of cyclic [^{3}H]AMP (0.25 pmol) were added to each supernatant, which was then purified over Dowex 50, and the cyclic AMP content quantified by the protein binding assay of Gilman [6].

3. RESULTS

We have presented evidence elsewhere that PGA, but not other prostaglandins, is effective in inhibiting cAMP efflux because of its capacity to form an adduct with cellular glutathione [4,5]. The electron-withdrawing effect of the 9-keto group in conjugation with the 10,11 double bond in the head group of PGA allows glutathione to attack at carbon 11 [7]. We reasoned that other molecules with a double bond in conjugation with a carbonyl or hydroxyl group or simply with conjugated double bonds in the carbon chain might also prove good candidates for attack by glutathione and thereby also inhibit export of cyclic AMP. In searching for such compounds, we found that many are insect pheromones. For instance, 9,11-myristic acetate is a pheromone for two noctuid moths, *Spodoptera littoralis* and *S. litura*, and 8,10-dodecadienol is a pheromone for the codling moth *Laspeyresia pomonella*. Both inhibit the rate of extrusion of cAMP from these cells (44 and 66%, respectively; fig.1).

Several compounds not expected to contain sites for glutathione attack (one or no double bonds) nonetheless also proved to be inhibitors of cyclic AMP export. Unsaturated fatty acids with 14, 16, 18, or 20 carbons, including prominent fatty acid constituents of membrane phospholipids, are good inhibitors of cyclic AMP export (table 1). Among these compounds, a few trends are apparent: (i) the presence of at least one double bond enhances the ability of the compound to inhibit cyclic AMP export; and (ii) given an identical carbon chain, the acid is a better inhibitor than the aldehyde. These trends are illustrated graphically in fig.2. Effective fatty acid/pheromone inhibitors of cyclic AMP efflux had EC_{50} values in the range of 5–50 μM (fig.3), in contrast to PGA_1 which has an EC_{50} of 45 nM.

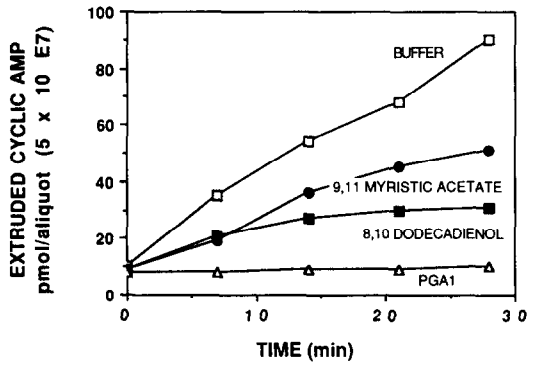

Fig.1. Pigeon red cells were preloaded with cyclic AMP by treatment with 10 μM isoproterenol, then washed and exposed to either 9,11-myristic acetate (pheromone for *Spodoptera littoralis* and *S. litura*) or 8,10-dodecadienol (pheromone for *Laspeyresia pomonella*) at 100 μM, or PGA_1 at 10 μM, and sampled at the indicated times for cAMP content of the media, as described in section 2. All cell suspensions contained the phosphodiesterase inhibitor, RO20-1724, at 50 μM.

Not all straight-chain hydrocarbons are effective (table 2). Dicarboxylic acids of 6–10 carbons are ineffective. Fatty alcohols with no or a single double bond in the carbon chain are only slightly effective. Acetate esters of 12 and 14 carbon compounds are also only partially inhibitory at 100 μM.

4. DISCUSSION

Inhibition of cyclic AMP export by pheromones is interesting in the context of hypotheses of odorant sensory transduction that involve cyclic nucleotides [8]. Although the concentrations required to inhibit cyclic AMP efflux in this system (avian red cells) seem high, this parameter could differ in specialized sensory cells. Such a mode of action could provide a modulatory role for pheromones and odorant lipids that may result from lipid-lipid interactions and may thus be distinct from putative interactions with olfactory receptor proteins.

The actions of various fatty acids to inhibit cyclic AMP efflux seems relevant to current studies of signal transduction in many systems: hormone and Ca^{2+}-activated phospholipases may generate substantial quantities of fatty acids within responsive cells, such that cyclic AMP export may be modulated as a result.

Table 1

Effective inhibitors of cyclic AMP export

Compound	Chain length	Position of double bond	End group	Inhibition (%) of cAMP export at 100 μM
trans-8,*trans*-10-Dodecadienol	12	C8,C10	OH	84
cis-9,*trans*-11-Tetradecadienol	14	C9,C11	OH	65
Myristic acid	14	0	COOH	72
Myristoleic acid	14	C9	COOH	88
Palmitic acid	16	0	COOH	27
Hexadecenal	16	C9	CHO	68
Palmitoleic acid	16	C9	COOH	82
Stearic acid	18	0	COOH	0
Oleic acid	18	C9	COOH	93
Linoleic acid	18	C9,C12	COOH	92
Linolenic acid	18	C9,C12,C15	COOH	98
Arachidonic acid	20	C5,C8, C11,C14	COOH	98
Eicosapentaenoic acid	20	C5,C8, C11,C14,C17	COOH	98

Efflux of cyclic AMP was monitored as described for figs 1,2 and the rates of export over 20 min were compared, untreated cells ('buffer' in fig.1) being 0% inhibition, cells treated with 10 μM PGA_1 being 100% inhibition. Data are means of one to three experiments

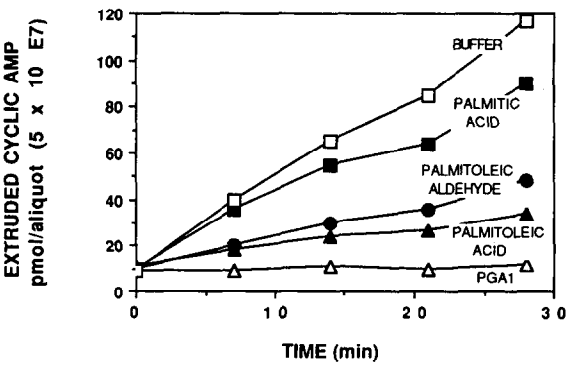

Fig.2. Effect of various fatty acids and aldehydes on cAMP extrusion; protocol is as described for fig.1. Note the improved inhibitory effect conferred by the presence of a double bond (palmitoleic acid vs palmitic acid) and the better inhibition of the acid rather than the aldehyde (palmitoleic acid vs palmitoleic aldehyde).

The two most potent compounds tested in this study were *trans*-10-dodecene-(1,9)-diol and the pheromone *trans*-8,*trans*-10-dodecadienol. With respect to length, charge distribution and position of double bonds, these compounds have similarities to carbons 1–12 of PGA (fig.4). Thus, these compounds are likely candidates for attack by cellular glutathione and could conceivably act in the manner of PGA. If this is the case, then the compounds must have poor affinity or low potency at the specific site of action of PGA-GSH. These compounds actually have concentration-response curves similar to those of the common membrane fatty acids (fig.3), in which either a single double bond or the spacing of multiple double bonds makes them unlikely candidates for attack by glutathione. In fact, prostaglandins B, E, and F, unable to form glutathione adducts [9], have dose-response curves very similar to those of the fatty acids shown in fig.3. It seems likely that the fatty acids and pheromones are not acting at the same site as PGA-GSH, but are inhibiting cAMP export by altering membrane fluidity and thereby affecting transport functions.

This more generalized effect seems reasonable in the light of known effects of fatty acids on membrane properties. Whole cells are able to incorporate externally applied fatty acids into their membranes in the range used in these experiments (1–100 μM) with subsequent changes in the fluid properties of the membrane [10]. Our data on the sharp temperature dependence of cAMP transport [2] indicates that the putative cAMP transport protein is sensitive to changes in lipid fluidity. Measurements of steady-state fluorescence

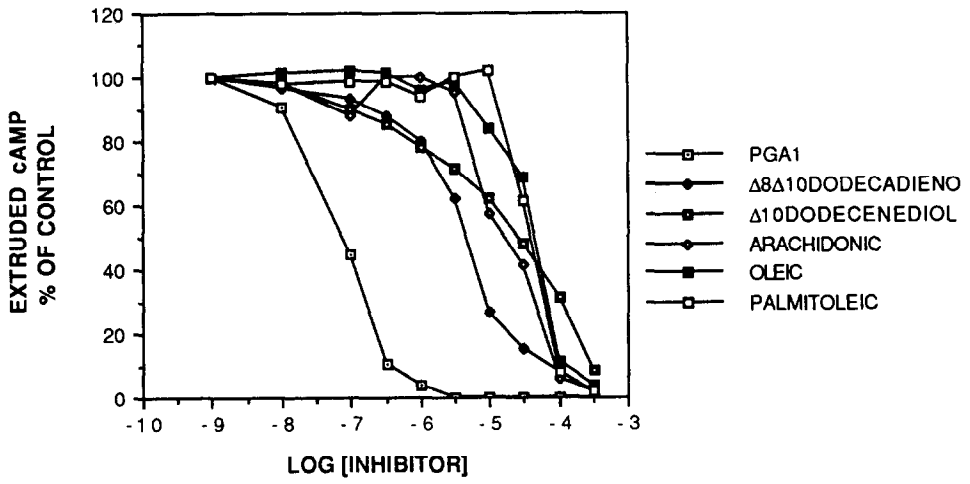

Fig.3. Pigeon red cells treated with isoproterenol for 10 min were washed and then exposed to the agents shown for 15 min, after which cAMP content of the media was determined.

Table 2

Relative ineffective inhibitors of cyclic AMP export

Compound	Chain length	Position of double bond	End group	Inhibition (%) of cAMP export at 100 μM
1,6-Hexanedioic acid	6	0	COOH	0
1,7-Heptanedioic acid	7	0	COOH	0
1,8-Octanedioic acid	8	0	COOH	0
1,9-Nonanedioic acid	9	0	COOH	0
1,10-Decanedioic acid	10	0	COOH	0
trans-5-Decen-1-ol	10	C5	OH	10
cis-7-Dodecen-1-ol	12	C7	OH	0
cis-8-Dodecen-1-ol	12	C8	OH	0
cis-7-Dodecenyl acetate	12	C7	O-CH_3COOH	14
trans-8,*trans*-10-Dodecadienyl acetate	12	C8,C10	O-CH_3COOH	30
Phenylheptanoic acid	13	C9,C11,C13	COOH	28
cis-9,*trans*-12-Tetradecadienyl acetate	14	C9,C12	O-CH_3COOH	18
cis-9-Tricosene	23	C9	CH_3	12

Data were collected as described for table 1

anisotropy reveal that the presence of one or more double bonds in the fatty acyl chain decreases order in the membrane and increases rate of motion and that this effect is most dramatic between a fully saturated hydrocarbon chain and the presence of just a single double bond; subsequent double bonds exert less additional effect [11]. The same impression is obtained from the data of table 1: the presence of one double bond enhances the inhibitory effect of a fatty acid; additional double bonds cause little additional effect. The parallel to the anisotropy data could suggest that if inhibition of transport is caused by a change in fluidity, it is via a decrease in order of the membrane, apparently resulting in a decreased interaction of a transport protein with cAMP or some other necessary molecule.

Many of the fatty acids that are effective in

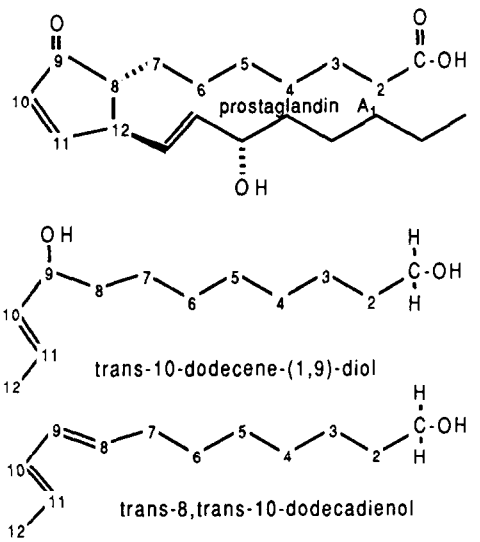

Fig.4. Size and charge distribution of two effective inhibitors of cyclic AMP efflux in relation to prostaglandin A_1.

blocking cAMP export (table 1) are common components of membrane phospholipids, some comprising large fractions of the total extractable membrane fatty acids. For example, in the S49 lymphoma cell, approximately one third by weight of total fatty acid is palmitic acid, with stearic, oleic, and linoleic acids representing about 15% each of membrane fatty acids [12]. Although estimation of the concentrations of fatty acids released from membranes during hormone-stimulated phospholipase activation is extremely difficult, it seems possible that the concentrations of free fatty acids in the vicinity of the membrane reach the micromolar concentrations required to inhibit the export of cyclic AMP.

The present data add another facet to the myriad of suggested inter-relations between phospholipid and cyclic AMP signaling pathways [13]. The products and sequelae of phosphoinositide turnover have the capacity either to enhance or to diminish the net results of the cyclic AMP pathway by actions at several different steps. The diacylglycerol and Ca^{2+} release resulting from phosphatidylinositol turnover can activate protein kinase C (the Ca^{2+}-stimulated phospholipid-dependent protein kinase), which can reduce β-receptor affinity for agonist, diminish receptor-G protein coupling, and yet enhance GTP-dependent catalytic activity of adenylate cyclase [14]. Unsaturated fatty acids (1 mM) may increase hormone-stimulated adenylate cyclase activity in either membranes or whole cells, presumably through increasing the fluidity of the membranes [15]. Unsaturated fatty acids in the range of 30–300 μM, although able to substitute in vitro for phosphatidylserine in the activation of protein kinase C, actually inhibit the in vitro activity of cAMP-dependent protein kinase [16]. Finally, the evidence presented here suggests that fatty acids in the 10–100 μM range can help maintain intracellular levels of cyclic AMP by blocking its export. All in all, we should consider fatty acids from membrane phospholipid metabolism as potential intracellular modifiers of the cyclic AMP pathway.

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