CONNECTIVE TISSUE ACTIVATION

IX. MODIFICATION BY PHARMACOLOGIC AGENTS

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Alpha- and beta-adrenergic blocking agents and imipramine inhibit the increased hyaluronate synthesis that may be induced in human synovial cultures by connective tissue activating peptide (CTAP). Considerations of drug concentration requirements, actions of analogues, and time studies all indicate that the adrenergic blockers do not act in this circumstance as conventional blockers of alpha or beta receptor sites. It is suggested that the membrane-stabilizing properties of these agents may be the important determinant for their limited "antiactivation" effect. Ethacrynic acid, a potent and more complete inhibitor of connective tissue activation, appears to act via a different mechanism.

Earlier reports described a connective tissue activating peptide (CTAP) that is extractable from human cells and capable of inducing increased metabolic activity in cultured synovial cells resembling the altered metabolic activity attributed to the synovial membrane in rheumatoid synovitis (1–4). CTAP has a molecular weight between 10,000 and 15,000 daltons, may be extracted from many cell types, and, when placed in contact with normal human synovial cells, induces metabolic consequences (*connective tissue activation*) including: *hyperproduction of low molecular weight hyaluronic acid, increased glucose uptake,* and *accelerated lactic acid production.* This constellation of phenomena clearly mimics those documented for rheumatoid synovitis in vivo, including the findings of excessive amounts of low molecular weight hyaluronic acid in joint fluid (5), decreased joint fluid PO_2 pH, and increased lactate concentrations (6). Studies showing that several commonly used antirheumatic drugs block connective tissue activation suggested that this in vitro system might be useful as a human model for specific components of the inflammatory process, and might be valuable for screening potential antiinflammatory agents and determining their site of action (7).

Although the mechanism of action of CTAP is not fully understood, current thinking of these authors is summarized in Figure 1, where CTAP is shown interacting with a complex cell membrane receptor site possessing at least two distinct components. At the bottom of the illustration the ultimate consequences of CTAP interaction with a synovial cell are

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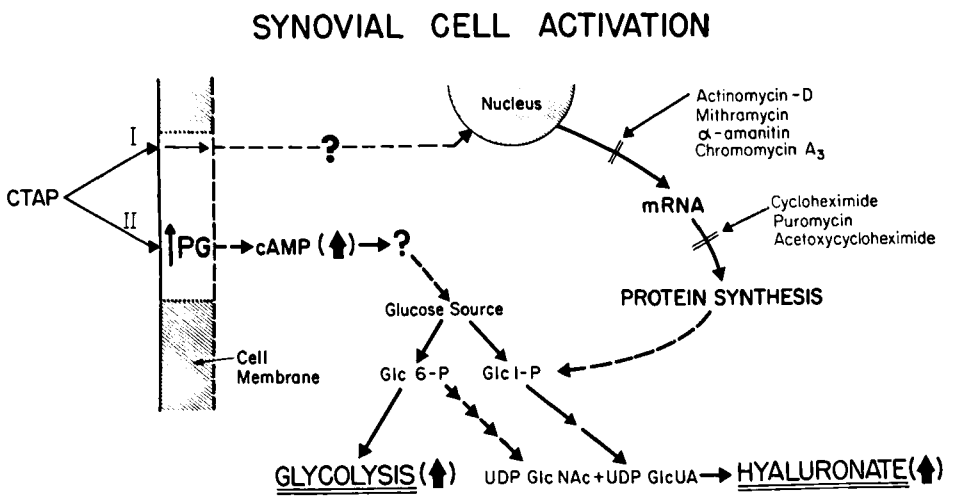

Fig 1. *This scheme suggests that activation of a synovial cell involves triggering two essential and independent reaction sequences leading ultimately to accelerated glycolysis and increased hyaluronic acid synthesis.*

shown as increased glycolysis and hyaluronic acid formation. A segment of the metabolic events following interaction of CTAP with a cell membrane receptor site may be visualized in relation to Component I where it has been shown that both mRNA synthesis and protein synthesis are required for the activation process, because the inhibitors of these events clearly block the action of CTAP (8). Component II of the receptor site mechanism may alter the synthesis of cellular prostaglandins, and the subsequent accumulation of synovial cell cyclic AMP may serve to mobilize energy and carbon for hyaluronate synthesis. Component II of the CTAP-synovial cell interaction is blocked by indomethacin (9); it is likely most of the other antiinflammatory organic acids act at this site.

In the context of this background, several pharmacologic agents, including adrenergic blocking agents, have been examined by the authors for their effect on the capacity of CTAP to stimulate human synovial cultures.

MATERIALS AND METHODS

Synovial membranes were obtained at arthrotomy or amputation and a portion of the tissue was fixed for histologic examination. The remainder of the tissue was divided into small fragments and used to generate cell cultures by methods reported previously (10). Cell strains used in this study were derived either from normal synovial membranes or from patients with degenerative joint disease or traumatic synovitis. Synovial cells from patients with these latter two entities had similar growth and biochemical parameters (10). Routine culture growth medium consisted of 80% synthetic medium 1066 (Grand Island Biological Company, Grand Island, New York), 10% fetal calf serum, and 10% heat-inactivated human serum, and was supplemented with L-glutamine, penicillin, and streptomycin. Human serum was inactivated by incubation at 56°C for 1 hour. Cells were enumerated and sized with a Coulter model B cell counter (Coulter Electronics, Hialeah, Florida). Synovial tissue fragments and suspensions of synovial fibroblast-like cells were stored by freezing with 6% dimethylsulfoxide in standard medium as reported earlier (10).

Culture protein was measured by the method of Oyama and Eagle (11), glucose by a glucose oxidase method (12), and lactate by the Barker-Summerson procedure (13). Hyaluronic acid in non-serum-containing medium was isolated using cetylpyridinium chloride without prior proteolysis (14), and uronic acid was determined by a carbazole method (15).

CTAP used in these experiments was prepared from normal human spleen, from HEp_{-2} tissue culture cells, and from normal human leukocytes by methods reported previously (16). Most of the CTAP preparations were heterogeneous by polyacrylamide gel electrophoresis.

In typical tissue culture experiments, 1×10^6 normal synovial cells were placed in T-15 flasks containing 2.0 ml of standard serum-containing medium. After 4 to 6 hours incubation at 37°C, the initial medium was replaced with 2 ml of Eagle's synthetic medium, buffered to pH 7.4 with 0.02 *M* Hepes (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, Calbiochem, Los Angeles, California) buffer supplemented with penicillin, streptomycin, and L-glutamine. Experimental flasks received 0.1 to 0.3 ml of test materials and control flasks received an equivalent volume of the appropriate vehicle. Vehicles utilized included 0.001 *M* dithiothreitol and 0.001 *M* cysteine. Flasks were incubated at 35–37°C for 40 hours and then measurements were made of total cell protein, total medium hyaluronic acid, total medium glucose, and lactic acid. The hyaluronic acid synthesis rate was expressed in micrograms of hyaluronic acid per milligram of cell protein per 24

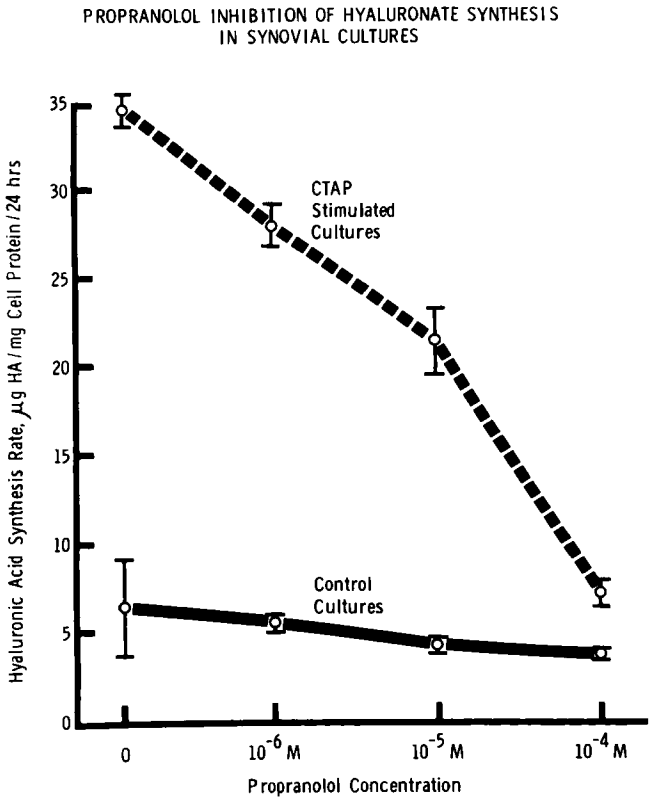

Fig 2. *dl-Propranolol exhibits a dose-dependent capacity to inhibit hyaluronate synthesis in CTAP-activated synovial cultures. Data are plotted as mean ± 1 SD.*

hours; glucose uptake and lactate formation were expressed in micromoles per milligram of cell protein per 24 hours. Experimental groups consisted of two to four flasks and the data were expressed in terms of means ± 1 SD.

Chlorpromazine, haloperidol, phenoxybenzamine, alprenolol, and practolol were obtained from commercial sources. Butoxamine (lot 47162) was a gift of the Burroughs Wellcome Company, Research Triangle Park, North Carolina. Phentolamine hydrochloride (K-6835) and imipramine were kindly provided by the Ciba Pharmaceutical Company, Summit, New Jersey. Propranolol hydrochloride (81-6474-1) was a gift from the Ayerst Laboratories, New York, New York. Albuterol (C5052) was provided by the Schering Corporation, Kenilworth, New Jersey, and L-isoproterenol hydrochloride was purchased from the Sigma Chemical Company, St. Louis, Missouri. Ethacrynic acid was a gift from Merck, Sharp and Dohme Research Laboratory, Rahway, New Jersey. The dimethylquaternary derivative of propranolol was made available by Dr. Benedict Lucchesi, Professor of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan.

RESULTS

Effects of Alpha- and Beta-Adrenergic Blocking Agents. As shown in Figure 2, propranolol, a $beta_{1,2}$ blocking agent, had a minor suppressive effect on hyaluronate formation in unstimulated synovial cultures. On the other hand, CTAP-stimulated cultures were progressively inhibited with respect to hyaluronate formation as propranolol was increased from 10^{-6} *M* to 10^{-4} *M*, and at the latter concentration formation of incremental hyaluronate was nearly abolished. Figure 3 shows that propranolol had little effect on CTAP-stimulated formation of lactic acid. On the other hand, in control cultures the highest concentrations of propranolol actually stimulated lactic acid formation. In the same vein, Figure 4 shows that CTAP-stimulated uptake of glucose was also virtually unaffected by propranolol. As with lactate production, the highest concentration of propranolol had a modest tendency to stimulate glucose uptake when used alone. The effects of alprenolol (a $beta_{1,2}$ blocking agent similar to propranolol) and butoxamine (primarily a $beta_2$ blocking agent) are depicted in Figure 5. The vehicle, alprenolol, and butoxamine, added singly, had no effect on incremental hyaluronate synthesis, whereas maximal stimulation was achieved with CTAP. Alprenolol (10^{-5} *M*) added with CTAP caused 50% inhibition of CTAP stimulation. The effect of increasing concentrations of butoxamine on CTAP stimulation is shown. Inhibition of CTAP stimulation was marked

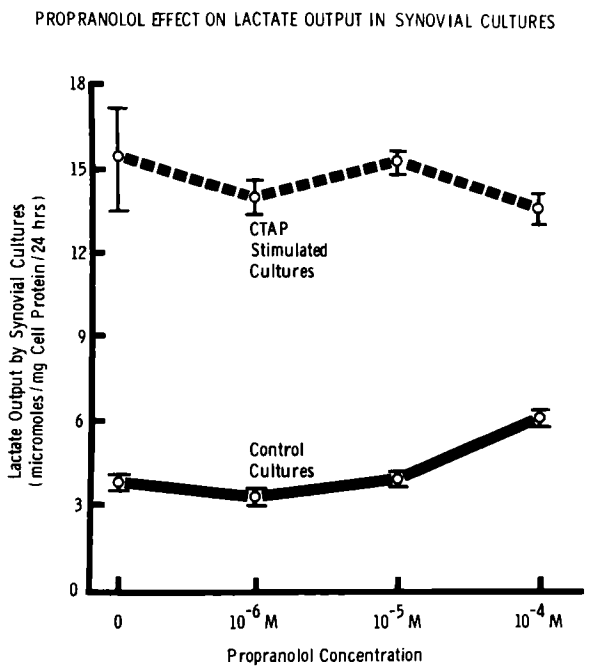

Fig 3. *dl-Propranolol did not suppress lactate formation in CTAP-activated synovial cultures.*

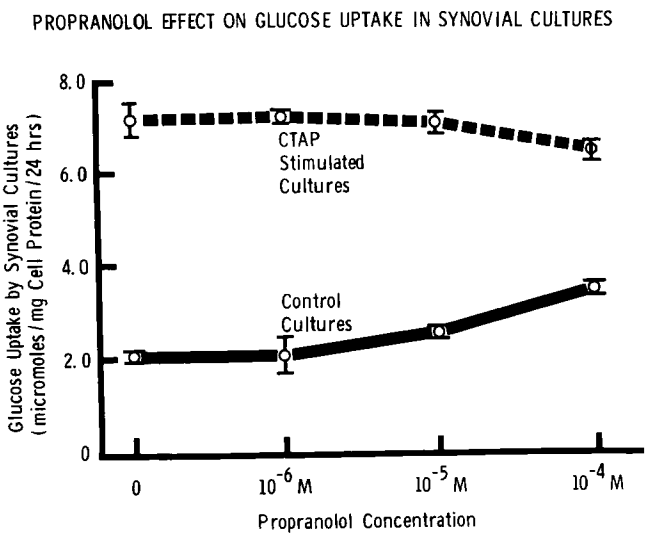

Fig 4. *Glucose consumption in CTAP-activated synovial cultures was not influenced by dl-propranolol.*

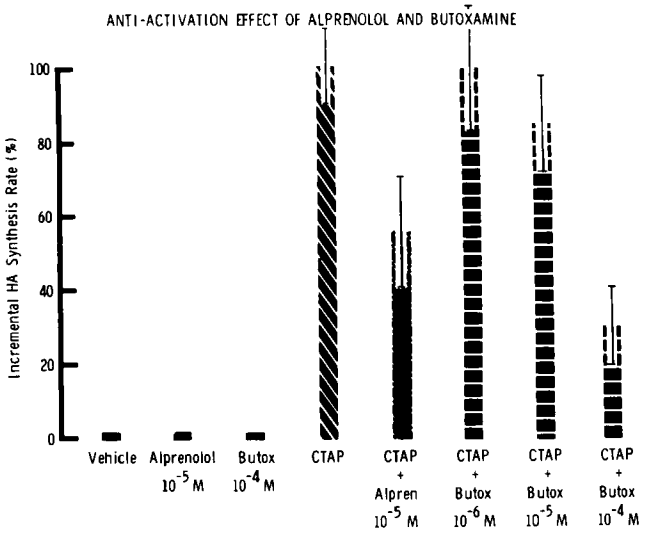

Fig 5. *Both alprenolol, a beta$_{1,2}$ adrenergic blocking agent, and butoxamine, a beta$_2$ blocking agent, markedly inhibit incremental hyaluronate synthesis in CTAP-activated synovial cultures. Incremental hyaluronate synthesis rate reflects that increase in HA synthesis (μgHA/mg cell protein/24 hours) caused by a stimulatory agent. In this figure and Figure 6, the agonist was CTAP and the increment in HA synthesis due to this agent was arbitrarily set at 100%.*

at 10^{-4} *M*, minimal at 10^{-5} *M*, and absent at 10^{-6} *M*.

Similar data concerning representative adrenergic blocking agents are exhibited in Figure 6. In this experiment, butoxamine (10^{-4} *M*) completely inhibited CTAP-induced incremental hyaluronate synthesis. Propranolol, the prototype beta$_{1,2}$ blocker, had the same inhibitory effect at 10^{-4} *M*, but interestingly practolol, a beta$_1$ blocking agent, had essentially no inhibiting effect on the activating capacity of CTAP. Of considerable interest was evidence that phentolamine, an alpha blocking agent, also was markedly inhibitory at 10^{-4} *M*. Other alpha blocking agents having a potent inhibitory effect on synovial cell activation included chlorpromazine, haloperidol, phenoxybenzamine, and several experimental alpha-adrenergic blocking agents. In summary, in the limited group of drugs tested, adrenergic agents with either beta$_2$ or alpha blocking activity inhibited CTAP activation of synovial cells at concentrations of 10^{-4} *M* to 10^{-5} *M*.

Because the concentrations of blocking agents required to inhibit synovial cell activation were so high (10^{-5} *M* to 10^{-4} *M*), it was suspected that something other than typical blockade of alpha- or beta-adrenergic receptor sites was involved in this pharmacologic action. Table 1 shows the inhibitory effect of propranolol given simultaneously with CTAP (at 0 time) and 16 hours following CTAP treatment of synovial cells. Propranolol alone had no effect on

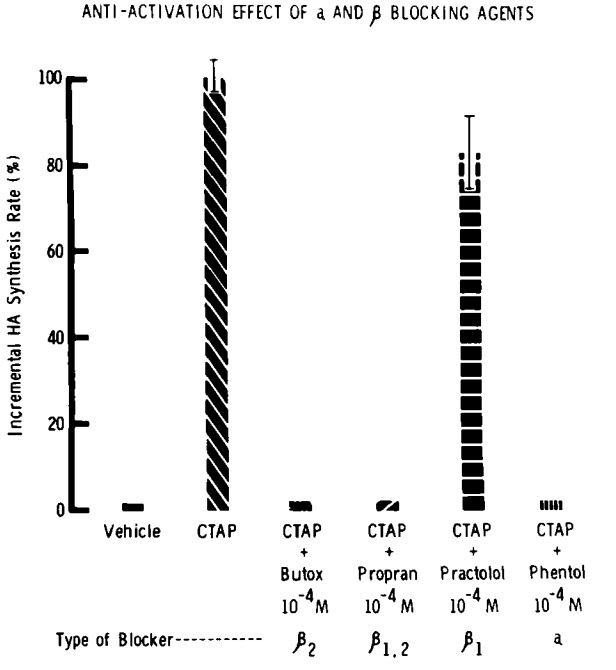

Fig 6. *Practolol, a beta$_1$ adrenergic blocking agent, was without effect on CTAP-activated synovial cultures, but beta$_{1,2}$, beta$_2$, and alpha blocking agents were markedly inhibitory.*

Table 1. *Effect of dl-Propranolol on Activated Synovial Cells*

Culture Additives	Time of Addition (hr)	Glucose Uptake (μmoles/mg cell protein/24 hr)	Lactate Output (μmoles/mg cell protein/24 hr)	Hyaluronate Synthesis Rate (μg/mg cell protein/24 hr)
Vehicle	—	0.18	4.03 ± 1.10	1.2 ± 0.8
Propranolol, 10^{-4} *M*	0	1.06 ± 0.45	3.27 ± 1.60	0.6 ± 0.5
Propranolol, 10^{-4} *M*	16	1.70 ± 0.84	2.56 ± 1.43	1.2 ± 1.4
CTAP*	0	3.74 ± 1.82	8.47 ± 0.95	57.2 ± 18.1*
CTAP + propranolol	0	2.71 ± 1.31	11.77 ± 1.23	18.0 ± 1.8
CTAP + propranolol	16	4.06 ± 1.49	12.33 ± 1.07	34.0 ± 4.9†

Fresh medium was added to normal synovial cell strain TC, ninth passage at 16 hours after addition of CTAP. dl-Propranolol was added at zero time to one group of activated flasks, and at 16 hours to a second group of activated flasks.
*CTAP was derived from HEp_{-2}.
†The difference is significant at $P < 0.05$.

hyaluronate synthesis, whereas CTAP caused a 47-fold increase in hyaluronate. Propranolol (10^{-4} *M*) added simultaneously with CTAP inhibited incremental hyaluronate synthesis by 70%. When propranolol was added 16 hours after CTAP, presumably long after the initiatory events at the receptor site, inhibition of incremental hyaluronate formation was still observed, although the effect was not as great as that at zero time. It thus appears that propranolol interferes with events subsequent to the initiating membrane phenomena. To clarify whether the adrenergic blocking agents acted after that early portion of the activation sequence involving prostaglandin and cyclic AMP, the effect of propranolol and chlorpromazine on stimulated hyaluronate synthesis caused by high concentrations of PGE_1 and dibutyryl cyclic AMP was examined (Figure 7). Control cultures synthesized minor amounts of hyaluronate, whereas PGE_1 and dibutyryl cyclic AMP stimulated substantial increments in hyaluronic acid. Propranolol and chlorpromazine both markedly depressed PGE_1 stimulation of synovial cells. In the same vein, both adrenergic blocking agents also suppressed dibutyryl cyclic AMP stimulation of hyaluronic acid synthesis in synovial cells.

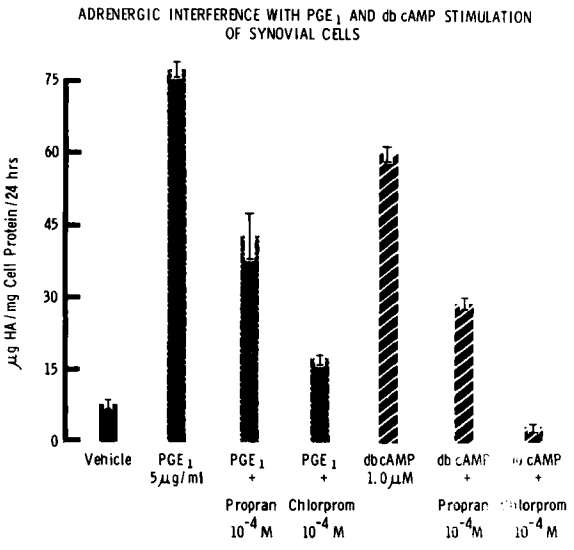

Fig 7. *Both PGE_1 and dibutyryl cyclic AMP in high concentrations stimulate synovial cell hyaluronate synthesis. This stimulation was effectively eliminated in both cases by dl-propranolol and chlorpromazine.*

Failure of Adrenergic Agonists to Stimulate Synovial Cells. Isoproterenol, a potent beta-adrenergic agonist, was added to synovial cultures in the abscence of serum at concentrations of 10^{-8} *M* and 10^{-4} *M* without measurable alteration of hyaluronate formation or glycolysis. Simultaneous addition of theophyllin (0.5×10^{-3} *M*) with isoproterenol also did not result in stimulation of synovial cells. Albuterol, a $beta_2$ agonist, failed to stimulate hyaluronate synthesis or glycolysis in concentrations of 4×10^{-3} *M* and 1.3×10^{-3} *M*. Epinephrine (1×10^{-4} *M*) was also ineffective in stimulating these parameters in human synovial cell cultures.

Relative Importance of Beta Blocking, Local Anesthetic, and Membrane Stabilizing Properties. Further evidence that the "antiactivation" effects of adrenergic blocking agents are not coupled to beta blocking ability is found in Table 2. These data show that d-propranolol, a relatively ineffective beta blocking agent (17), is similar to dl-propranolol in its capacity to inhibit synovial cell activation. On the other hand, UM-272 (a dimethyl quaternary derivative of propranolol) was a weaker inhibitor of synovial cell activation. In three further experiments the "antiactivation" potency of UM-272 ranged from zero to

Table 2. *Effect of dl-Propranolol, d-Propranolol, and a Quaternary Propranolol Derivative on Synovial Cell Activation*

Additives	Glucose Uptake (μmoles/mg cell protein/24 hr)	Lactate Output (μmoles/mg cell protein/24 hr)	Hyaluronate Synthesis Rate (μg/mg cell protein/24 hr)
Vehicle	3.70 ± 0.14	4.30 ± 0.68	9.7 ± 0.4
dl-Propranolol, 10^{-4} *M*	4.71 ± 0.66	6.87 ± 0.65	7.6 ± 0.4
d-Propranolol, 10^{-4} *M*	4.83 ± 0.13	7.91 ± 1.33	8.2 ± 0.5
UM-272, 10^{-4} *M*	4.00 ± 0.72	5.81 ± 0.91	8.5 ± 1.5
CTAP	7.40 ± 0.14	12.82 ± 2.37	20.2 ± 1.7
CTAP + dl-propranolol	6.70 ± 0.47	11.67 ± 2.09	12.5 ± 1.8*
CTAP + d-propranolol	6.79 ± 0.21	12.74 ± 0.56	11.4 ± 0.2*
CTAP + UM-272	7.65 ± 0.15	13.26 ± 1.67	16.5 ± 2.3†

*Depression below activated (CTAP) values was significant at $P < 0.01$.
†The minor depression in activation in the presence of UM-272 was significant at $P < 0.05$.

minimal. These observations are of interest because UM-272, although possessing antiarrhythmic properties, does not have demonstrable beta-adrenergic receptor blocking or local anesthetic activity (18).

Because it seemed unlikely that the adrenergic blocking agents exerted their effect on activation by conventional blockade of alpha and beta receptor sites, and because these agents are known to have potent membrane stabilizing effects (local anesthetic effects), model experiments were carried out with procaine and lidocaine, both potent local anesthetics characterized by marked membrane stabilizing activity. Neither procaine nor lidocaine was a very effective agent in suppressing CTAP activation of synovial cells (Figure 8). CTAP caused a threefold increase in hyaluronate synthesis and procaine (10^{-3} *M*) had little suppressive effect. Lidocaine (10^{-5} *M* and 10^{-4} *M*) had no inhibitory effect and was only moderately inhibitory at 10^{-3} *M*. Thus procaine and lidocaine were only one-tenth as potent as dl-propranolol and other adrenergic-blocking agents in opposing the activation process.

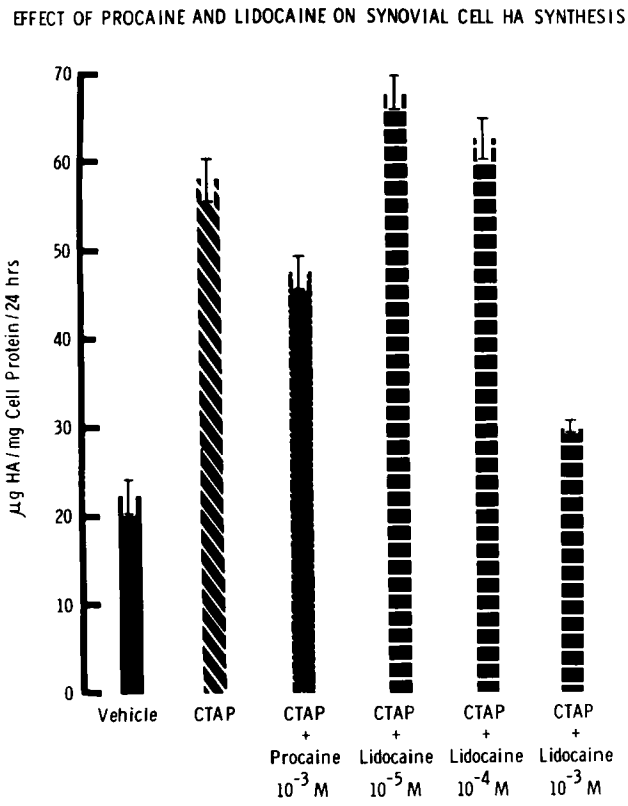

Fig 8. *Although both procaine and lidocaine inhibit CTAP-activated synovial culture synthesis of hyaluronic acid, ten times as much drug is required as in the case of propranolol and the other adrenergic blocking agents.*

Actions of Imipramine and Ethacrynic Acid. Imipramine, an antidepressant with marked local anesthetic properties, was a potent inhibitor of synovial cell activation. As shown in Table 3, incremental hyaluronate following either CTAP or dibutyryl cAMP was markedly reduced by imipramine, 10^{-4} *M*. Glucose uptake and lactate formation were stimulated by imipramine added alone, but the drug did not modify CTAP alteration in these parameters.

Ethacrynic acid (0.83×10^{-4} *M*) resembled imipramine in that addition of the drug at zero time blocked incremental hyaluronate synthesis in synovial cultures stimulated by either CTAP or dibutyryl cAMP (Table 4). Unlike imipramine and the adrenergic blocking agents, ethacrynic acid interfered with CTAP-stimulated glycolysis. An experiment showing the effect of time of addition of three drugs is shown in Table 5. When dl-propranolol, imipramine, and ethacrynic acid were added simultaneously with

Table 3. *Effect of Imipramine on Synovial Cell Activation*

Culture Additives	Glucose Uptake (μmoles/mg cell protein/24 hr)	Lactate Output (μmoles/mg cell protein/24 hr)	Hyaluronate Synthesis Rate (μg/mg cell protein/24 hr)
Vehicle	3.28 ± 0.14	4.22 ± 0.14	7.5 ± 0.2
Imipramine, 10^{-4} *M*	4.27 ± 0.22	7.06 ± 0.80	6.5 ± 1.1
CTAP	4.75 ± 0.06	7.40 ± 0.27	16.2 ± 1.6
CTAP + imipramine, 10^{-4} *M*	4.87 ± 0.15	8.43 ± 0.82	7.2 ± 0.2
Db cAMP, 0.8 mM	3.60 ± 0.21	4.30 ± 0.27	97.8 ± 4.5
Db cAMP, 0.8 mM + imipramine, 10^{-4} *M*	4.27 ± 0.23	8.21 ± 0.48	26.4 ± 1.7

Normal cell strain ACS, sixth passage was the target culture. CTAP was derived from the HEp_{-2} cell strain. Data are expressed as mean ± 1 SD.

CTAP to synovial cultures, there was 67%, 94%, and 57% suppression, respectively, of incremental hyaluronate over the first 16 hours. Media were changed and the inhibitory effect of propranolol and imipramine decreased in the ensuing 24 hours. Ethacrynic acid treated cultures, on the other hand, showed progressive inhibition of incremental hyaluronate synthesis, even with omission of the drug. Addition of all three drugs 16 hours after CTAP led to a 50–76% inhibition of incremental hyaluronate synthesis in activated cultures, providing further evidence that these agents exert some inhibitory action subsequent to the initiating membrane effects.

DISCUSSION

Many pharmacologic agents used in this study inhibited primarily the incremental hyaluronate synthesis component of synovial cell activation and had little or no effect on CTAP-accelerated glycolysis. Beta-adrenergic blocking agents with this capacity included dl-propranolol, d-propranolol, alprenolol, and butoxamine. Alpha-adrenergic blocking agents that inhibited incremental hyaluronate synthesis included phentolamine, chlorpromazine, haloperidol, and phenoxybenzamine. Although beta-adrenergic blocking agents had little capacity to inhibit CTAP-stimulated glycolysis, in higher concentrations (1×10^{-4} *M*), they did modestly *stimulate* glucose consumption and lactate formation in their own right. In like manner, alpha-adrenergic blocking agents failed to block the glycolytic component of synovial cell activation. Imipramine, an antidepressant drug, resembles the phenothiazine drugs in many of its actions (19). When added to cultured human synovial cells, imipramine (1×10^{-4} *M*) blocked CTAP-induced incremental hyaluronate synthesis, but not incremental glycolysis. As with adrenergic blocking agents, it modestly stimulated glucose uptake and lactate formation when added alone to synovial cultures.

Ethacrynic acid (0.5 to 1.0×10^{-4} *M*) resembled conventional antirheumatic drugs in that it markedly suppressed both CTAP-induced increments

Table 4. *Effect of Ethacrynic Acid on Synovial Cell Activation*

Additives	Glucose Uptake (μmoles/mg cell protein/24 hr)	Lactate Output (μmoles/mg cell protein/24 hr)	Hyaluronic Acid Synthesis Rate (μgHA/mg cell protein/24 hr)
Vehicle	3.14 ± 0.28	9.90 ± 0.23	13.5
CTAP*	10.90 ± 2.18	22.39 ± 4.06	40.4 ± 7.7
CTAP + ethacrynic acid, 0.83×10^{-4} *M*	4.36 ± 2.37	13.83 ± 1.70	1.4 ± 1.3
Vehicle†	1.56 ± 0.20	4.13 ± 0.16	7.7 ± 0.6
Dibutyryl cAMP, 0.8 mM	3.74 ± 0.18	7.06 ± 0.12	60.9 ± 1.8
Ethacrynic acid 0.83×10^{-4} *M*	2.25 ± 0.24	6.31 ± 0.58	4.5 ± 0.4
Ethacrynic acid 0.83×10^{-4} *M* + dibutyryl cAMP, 0.8 mM	2.76 ± 0.17	7.92 ± 0.29	4.6 ± 0.2

Data are expressed as the mean ± SEM of quadruplicate observations.
If both Db cAMP and ethacrynic acid were used, they were added nearly simultaneously.
*CTAP used in each flask was extracted from 10^6 normal human leukocytes. The target culture was normal synovial cell strain ACS, fourth subculture. CTAP and drugs were added in that sequence with only minutes intervening between the agents.
†The target culture for this assay was the normal synovial cell strain RN, sixth subculture.

Table 5. *Effect of Time of Addition on "Antiactivating" Capacity of Propranolol, Imipramine, and Ethacrynic Acid*

Culture Additives	Time of Addition of Drug (hr)	Hyaluronic Acid Synthesis Rate (μg/mg cell protein/24 hr) First Time Period (0–16 hr)	Percent Suppression	Second Time Period (16–40 hr)	Percent Suppression
Vehicle	—	34.7 ± 5.7	—	22.7 ± 1.3	—
Propranolol, 10^{-4} *M*	0	25.5 ± 0	—	14.4 ± 3.2	—
Imipramine, 10^{-4} *M*	0	20.5 ± 3.1	—	16.8 ± 3.1	—
Ethacrynic acid, 0.5×10^{-4} *M*	0	21.5 ± 1.9	—	8.5 ± 0.7	—
CTAP*	0	63.4 ± 4.8	0	92.6 ± 10.3	0
CTAP + propranolol, 10^{-4} *M*	0	41.2 ± 4.8	67	71.5 ± 7.2	30
	16	71.9 ± 0.9	—	59.4 ± 10.6	47
CTAP + imipramine, 10^{-4} *M*	0	36.5 ± 7.2	94	45.0 ± 6.8	68
	16	58.5 ± 6.4	—	39.3 ± 8.9	76
CTAP + ethacrynic acid, 0.5×10^{-4} *M*	0	46.9 ± 3.4	57	13.1 ± 4.3	100
	16	58.2 ± 4.1	—	39.3 ± 4.1	76

Medium was removed from all cultures at 16 hours (first time period), completely replaced, and then removed again 24 hours later (second time period). Drugs were added at zero time (along with CTAP) or at the 16-hour point. Clearly, the inhibitory agents blocked activation whether added early or late, and in the former case the effect was not reversed by changing the medium and omitting the drugs.

*CTAP was always added at zero time. The preparation was derived from the non-erythroid cells of dog spleen.

in hyaluronate synthesis and glycolysis. When imipramine and propranolol-treated cultures promptly escaped from drug effects on omission of the agents, ethacrynic acid-induced suppression persisted for at least 24 hours after the culture was changed with drug-free media.

Procaine and lidocaine were studied because they share the "membrane-stabilizing" and local anesthetic properties found with adrenergic blocking agents, although lacking specific receptor site blocking properties. Higher concentrations (1×10^{-3} *M*) of these local anesthetics were required to inhibit CTAP-induced incremental hyaluronate synthesis than was the case with adrenergic blocking agents (1×10^{-5} *M* to 1×10^{-4} *M*). The dimethyl quaternary derivative of propranolol (UM-272), which lacks adrenergic blocking and local anesthetic activity, is also a very weak inhibitor of synovial cell activation.

Some of the known properties of these drugs and their efficacy as inhibitors of synovial cell activation are compared in Table 6. It seems clear that the "antiactivation" effect of the various drugs is unrelated to their adrenergic blocking properties, because alpha, $beta_{1,2}$, $beta_2$ agents, and some drugs without adrenergic activity, are equally effective in blocking CTAP-induced incremental hyaluronate synthesis. Furthermore, the concentrations required to inhibit incremental hyaluronate synthesis were several orders of magnitude higher than would be expected for classic adrenergic receptor site blockade. Lastly, it was clear that the agents could be added to synovial cultures several hours after the CTAP agonist, presumably long after cell membrane receptor site initiatory phenomena, and still inhibit CTAP-induced incremental hyaluronate synthesis. The argument that beta-adrenergic blocking agents act by interfering with the generation of intracellular cyclic AMP appears to be rendered moot by these observations. However, if one entertains a model wherein hormone (or other agonist) receptors exist separately from the catalytic site, there might be direct inhibition of adenyl cyclase analogous to that reportedly caused by phenothiazines in thyroid, adrenal, and hepatic tissue (20). It seems unlikely, however, that inhibition of adenyl cyclase (and consequent reduction in intracellular cyclic AMP) is a critical factor in the pharmacologic effects observed in the synovial culture system described here. In support of this position are the observations that dl-propranolol inhibited both PGE_1 and dibutyryl cyclic AMP-stimulated hyaluronate synthesis in synovial cultures, and that imipramine and ethacrynic acid depressed dibutyryl cyclic AMP-stimulated hyaluronate formation. In summary, it appears that most of the pharmacologic agents studied may act late in the synovial activation sequence. This late action may interfere with the synthesis of hyaluronate, or conceivably accelerate its degradation.

Most of the agents capable of inhibiting CTAP-induced incremental hyaluronate synthesis have local anesthetic and membrane-stabilizing activity. There is thought to be a close correlation between the ability of agents to stabilize erythrocytes against hypotonic hemolysis and their potency as local anesthetics (21). Biphasic patterns of stabilization and lysis have been

Table 6. *Comparison of Several Pharmalogic Agents*

Drug	Adrenergic Blocking Activity	Local Anesthetic Activity	Membrane Stabilizing Activity	Antiinflammatory Activity In Vivo	Concentration Required to Inhibit CTAP-Induced Incremental HA
dl-Propranolol	$\beta_{1,2}$	+	+	+	1×10^{-4} *M*
d-Propranolol	0	+	+	—*	1×10^{-4} *M*
UM-272	0	0	+	—	1×10^{-4} *M*†
Alprenolol	$\beta_{1,2}$	+	+	—	1×10^{-4} *M*
Practolol	β_1	0	0	—	No effect
Butoxamine	β_2	+	+	—	1×10^{-4} *M*
Phentolamine	α	0	+	—	1×10^{-4} *M*
Chlorpromazine	α	+	+	—	5×10^{-6} *M*
Imipramine	0	+	+	+	1×10^{-4} *M*
Ethacrynic acid	None	0	0	+	5×10^{-5} *M*
Procaine	0	+	+	—	1×10^{-3} *M*
Lidocaine	0	+	+	—	1×10^{-3} *M*

*A dash indicates that no data were available.
†Minor effect.

demonstrated for several local anesthetics, alcohols, steroid hormones, and phenothiazines using erythrocyte and lysosomal membranes as indicator systems (21). Chlorpromazine for example shows activity as a stabilizer of lysosomal membranes starting near 5×10^{-5} *M*, and reaches a maximum near 5×10^{-4} *M*, with rapid impairment of membrane integrity (lysis) at slightly higher concentrations. The chlorpromazine concentration required to inhibit CTAP-induced incremental hyaluronate formation corresponds closely with that required to provide maximum membrane stabilization. Membrane stabilization involves changes in physical properties, including increased mechanical strength, expansion, and altered permeability with decreased transmembranal flux of sodium and potassium (21). In view of the likely importance of membrane-associated steps in hyaluronic acid biosynthesis, it is perhaps not unreasonable to expect agents acting at this level to interfere with hyaluronate synthesis.

Ethacrynic acid appears to be set apart from the other agents studied, not only providing more complete inhibition of activation effects, but being less easily reversible. Diverse actions of ethacrynic acid that have been reported include: inhibition of potassium activated *p*-nitrophenylphosphatase and certain ATPases in normal white blood cells (22), inhibition of cholera exotoxin-induced secretion of gut electrolytes (23), and inhibition of the inflammatory process (24). Studies on cotton pellet granulomas in rats indicated that ethacrynic acid substantially depressed granuloma weight (24). The reversal of this antiinflammatory effect with cysteine was thought to support a mechanism of action involving interaction with sulfhydryl groups. Among the other agents studied here, at least two have been shown to possess antiinflammatory activity in vivo. Imipramine inhibits paw edema in the rat and inflammatory reactions induced by formalin, egg white, and croton oil (19). Propranolol in doses of 0.5 to 2.5 mg/100 g appeared to inhibit formaldehyde-induced arthritis in rats (25). With the perspective provided by the foregoing observations, it may be useful to observe carefully patients treated with these pharmacologic agents for evidence of altered connective tissue metabolism and response to inflammatory stimuli.

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REFERENCES

1. Yaron M, Castor CW: Leukocyte-connective tissue cell interaction. I. Stimulation of hyaluronate synthesis by live and dead leukocytes. Arthritis Rheum 12:365–373, 1969
2. Castor CW, Yaron M: Leukocyte-connective tissue cell interaction. II. The specificity, duration, and mechanism of interaction effects. Arthritis Rheum 12:374–386, 1969
3. Castor CW: Connective tissue activation. I. The nature, specificity, measurement and distribution of connective tissue activating peptide. Arthritis Rheum 14:41–54, 1971
4. Castor CW: Connective tissue activation. II. Abnormalities of cultured rheumatoid synovial cells. Arthritis Rheum 14:55–66, 1971
5. Castor CW, Prince RK, Hazelton MJ: Hyaluronic acid in human synovial effusions: a sensitive indicator of altered connective tissue cell function during inflammation. Arthritis Rheum 9:783–794, 1966

6. Falchuk KH, Goetzl EJ, Kulka JP: Respiratory gases of synovial fluids. An approach to synovial tissue circulatory-metabolic imbalance in rheumatoid arthritis. Am J Med 49:223–231, 1970
7. Castor CW: Connective tissue activation. IV. Regulatory effects of antirheumatic drugs. Arthritis Rheum 15:504–514, 1972
8. Castor CW: Connective tissue activation. III. Observations on the mechanism of action of connective tissue activating peptide. J Lab Clin Med 79:285–301, 1972
9. Castor CW: Connective tissue activation. VII. Evidence supporting a role for prostaglandins and cyclic nucleotides. J Lab Clin Med 85:392–404, 1975
10. Castor CW: Abnormalities of connective tissue cells cultured from patients with rheumatoid arthritis. II. Defective regulation of hyaluronate and collagen formation. J Lab Clin Med 77:65–75, 1971
11. Oyama VI, Eagle H: Measurement of cell growth in tissue culture with a phenol reagent (Folin-Ciocalteau). Proc Soc Exp Biol Med 91:305–307, 1956
12. Gibson QH, Swoboda BEP, Massey V: Kinetics and mechanism of action of glucose oxidase. J Biol Chem 239:3927–3934, 1964
13. Barker SB, Summerson WH: The colorimetric determination of lactic acid in biological material. J Biol Chem 138:535–554, 1941
14. Castor CW, Wright D, Buckingham RB: Effects of rheumatoid sera on fibroblast proliferation and hyaluronic acid synthesis. Arthritis Rheum 11:652–659, 1968
15. Bitter T, Muir HM: A modified uronic acid carbazole reaction. Anal Biochem 4:330–334, 1962
16. Castor CW: Connective tissue activation. VI. The effects of cyclic nucleotides on human synovial cells *in vitro*. J Lab Clin Med 83:46–55, 1974
17. Lucchesi BR, Whitsitt LS, Stickney JL: Antiarrhythmic effects of beta-adrenergic blocking agents. Ann NY Acad Sci 139:940–941, 1967
18. Schuster DP, Lucchesi BR, Nobel NL, et al: The antiarrhythmic properties of UM-272, the dimethyl quaternary derivative of propranolol. J Pharmacol Exp Ther 184:213–227, 1973
19. Gyermels L: The pharmacology of imipramine and related antidepressants. Int Rev Neurobiol 9:95–143, 1966
20. Wolff J, Jones AB: Inhibition of hormone-sensitive adenyl cyclase by phenothiazines. Proc Natl Acad Sci USA 65:454–459, 1970
21. Seeman PM: Membrane stabilization by drugs: tranquilizers, steroids, and anesthetics. Int Rev Neurobiol 9:146–221, 1966
22. Rao GJS, Del Monte M, Nadler HL: A K^+-activated, ethacrynic acid-sensitive p-nitrophenylphosphatase from normal human white cells. Biochem Biophys Acta 229: 454–457, 1971
23. Carpenter CCJ, Curlin GT, Greenough WB: Response of canine thiry-vella jejunal loops to cholera exotoxin and its modification by ethacrynic acid. J Infect Dis 120:332–338, 1969
24. Oronsky AL, Triner L, Steinsland OS, et al: Effect of sulphydryl binding compounds on the inflammatory process. Nature 223:619–621, 1969
25. Jaju BP, Sinha JN, Sharma HN: Experimental studies with propranolol as a local anaesthetic. Br J Anaesth 41:594–596, 1969