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INFLUENCE OF HYDROCORTISONE ON THE MODULATION OF THE INFLAMMATORY RESPONSE

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Summary.—*In vitro* hydrocortisone, in pharmacologically attainable concentrations, binds nonspecifically to rat peritoneal mast cells and amplifies the stimulating effects of PGE_1 on membrane-bound adenylate cyclase. As a consequence, the intracellular concentration of cyclic AMP in the target cells increases and histamine release is markedly reduced. Deoxycorticosterone, at the same concentrations, has no effect. These findings may in part explain the mechanism of action of anti-inflammatory steroids, possibly related to the modulating effects of E-type prostaglandins.

ALTHOUGH considerable attention has been devoted to the anti-inflammatory activity of corticosteroids, very little is known yet about the mechanisms underlying these effects. The early suggestion of corticosteroids acting as membrane stabilizers has been questioned (Lewis, Symons and Ancill, 1974; Persellin and Ku, 1974) but, in any case, it is not clear precisely what is meant by "stabilization". More recently, Lewis and Piper (1975), on the basis of their study on the infusion of adrenocorticotrophic hormone into rabbit adipose depots, have suggested that inhibition of prostaglandin release from tissues may account for some of the anti-inflammatory effects of corticosteroids. This view has been largely confirmed (Kantrowitz *et al.*, 1975; Tashjian *et al.*, 1975; Hong and Levine, 1976), triggering an increasing series of investigations on the control of prostaglandin synthesis and its possible relationship to the mechanism of action of anti-inflammatory steroids (De Asua *et al.*, 1977; Saeed *et al.*, 1977).

Since E-type prostaglandins (PGE) may regulate the character and the intensity of the inflammatory response through adenylate cyclase activation (Bourne *et al.*, 1974) and since the first step in steroid action is the binding of the hormone to membrane receptors (Munck and Brink-Johnsen, 1968; Baxter *et al.*, 1971; Lippman and Barr, 1977), it seemed worthwhile considering that corticosteroids might interfere with prostaglandins functioning as "stop-signal" effectors.

In the present report we provide evidence that hydrocortisone amplifies the modulating effects of PGE_1 leading to the suppression of histamine release by rat peritoneal mast cells.

MATERIALS AND METHODS

Reagents.—Reagents and their sources were as follows: Adenosine 3′:5′ cyclic monophosphoric acid (cAMP), bovine serum albumin (BSA), hydrocortisone 21-sodium succinate (Hyc), 11-deoxycorticosterone (Doc) and Compound 48/80 were purchased from Sigma Chemical Co.; cortisol-4-^{14}C (50 mCi/mmol) and deoxycorticosterone-1,2-^{3}H (36 Ci/mmol) from Amersham Radiochemicals; prostaglandin E_1 was a gift from Dr G. Nisticò; all other reagents were obtained from Fisher Scientific. Radioactive steroids were used as purchased, usually within a few days of receipt, and their molar concentrations were determined from the nominal specific activities.

Mast cell preparations.—The methods of collection and purification of mast cells were as described previously (Tolone, Bonasera and

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Tolone, 1978). Briefly, male Sprague–Dawley rats (200 to 250 g) were anaesthetized with ether, exsanguinated by cutting the carotid arteries, and laparotomized; peritoneal cells were harvested in a medium containing 150 mM NaCl, 3·7 mM KCl, 3·0 mM Na_2HPO_4, 3·5 mM KH_2PO_4, 0·9 mM $CaCl_2$, 5·6 mM dextran, 0·1% (w/v) BSA, 0·1% gelatin and 10 u/ml heparin (pH 6·8). After purification by a BSA density gradient centrifugation method (Sullivan *et al.*, 1975*b*), mast cells of 90% or greater purity were obtained. Cell concentration, V (ml of packed cells per ml of cell suspension), was determined by a standard microhaematocrit procedure and cell viability was checked by trypan blue exclusion (van Furth and van Zwet, 1973).

Incubation conditions.—In most experiments, 1×10^6 isolated mast cells in 0·5 ml of medium were incubated with equal volumes of medium containing or not containing hydrocortisone or deoxycorticosterone at the indicated concentrations. Incubations were carried out at 37° for 15 min before adding 10 nM or 10 uM PGE_1. At the end of an additional 15 min incubation period, the tubes were placed in ice-water and centrifuged (1200 *g*) at 4° for 10 min. In some experiments, cells were exposed simultaneously to hydrocortisone and PGE_1, then incubated for 15 min at 37°, cooled and centrifuged as mentioned. The supernatant solutions and cell pellets were chilled and stored at −80° for later histamine and cAMP determinations. When appropriate, Compound 48/80 at a final concentration of 1 μg/ml was added to each sample 2 min before cooling.

Cyclic AMP assay.—Specimens (frozen pellets or aliquots of supernatants) were reconstituted in 50 mM sodium acetate buffer, pH 6·2, heated at 104° for 10 min, sonicated and centrifuged at 1800 *g* for 10 min. The clear supernatants were then transferred to fresh tubes and tested for cAMP content by radioimmunoassay. The Schwarz–Mann cAMP–RIA set and the procedures supplied with the kit were used. Standard curves were run with each assay and were found to be consistently linear. Duplicate samples were generally tested and the final results were expressed as pmoles cAMP per 10^6 mast cells.

Histamine assay.—Histamine was determined in cell supernatants and pellets by the o-phthalaldehyde spectrofluorometric procedure of Shore (1971), described in detail elsewhere (Tolone, Bonasera and Parrinello, 1974). Total histamine was measured, as reported by Sullivan, Green and Parker (1975*a*), on cell samples which had been boiled and depleted of protein by the addition of a one-tenth volume of 70% perchloric acid followed by centrifugation. The data were expressed in terms of per cent histamine release.

Binding experiments.—The physico-chemical interactions of hydrocortisone or deoxycorticosterone with isolated mast cells were investigated according to the procedures described by Munck and Brinck-Johnsen (1968). From a mast-cell suspension (V range 0·15–0·30) incubated (37°) with a radioactive steroid for a suitable time interval (usually 20 min), a 50 μl aliquot was removed by suction and counted to give the value T (counts/min/50 μl). The suspension was then centrifuged for 2 min in a Beckman Microfuge and from the supernatant a 50 μl sample was counted to give S (counts/min/50 μl). Defining (S) as the steroid concentration in the medium (the aforementioned salt-balanced solution lacking BSA and gelatin), and (Sc) as the steroid bound to the cells (moles/l of packed cells) it follows that $(Sc)e/(S)e = T - S(1-V)/VS$, where e denotes equilibrium conditions. The variation in $(Sc)e/(S)e$ among repeated samples from one preparation was usually in the range of 10%. Variations among preparations were sometimes higher than 30%. When appropriate, preincubated cell suspensions were diluted about 50-fold and reincubated as before. Beginning immediately after dilution, samples were removed at the indicated time intervals and centrifuged to obtain cell pellets and supernatants for assay. The ratio for expressing dissociation results is $(Sc)d/(S)e$, where $(Sc)d$ indicates the concentration of steroid bound to the cells after dilution. $(S)e$ is the same value as the one used for expressing equilibrium results, so that zero time value for $(Sc)d/(S)e$ is the ratio $(Sc)e/(S)e$ obtained before dilution.

Statistical analysis.—Differences from control values were analysed by Student's t test. Comparisons were of independent mean values.

RESULTS

Binding of hydrocortisone and deoxycorticosterone to rat peritoneal mast cells

Isolated rat peritoneal mast cells incubated at 37° for a suitable time interval (20 min) in the presence of isotopically labelled hydrocortisone (^{14}C-Hyc) or deoxycorticosterone (^{3}H-Doc) bind radioactive hormones to their plasma membrane. The magnitude of steroid binding seems to be not correlated with the anti-inflammatory activity of corticosteroids, since inactive deoxycorticosterone is bound much more than active hydrocortisone. In fact, as shown in Table I, binding of ^{3}H-Doc to mast cells at equilibrium ($(Sc)e/(S)e$) is approximately three times higher than the one of ^{14}C-Hyc, regardless of the initial free steroid concentration.

TABLE I.—*Rates of Hydrocortisone and Deoxycorticosterone Binding to Rat Peritoneal Mast Cells*

Steroids	Molarity (M)	Binding ratio			
		$(Sc)e/(S)e$ zero time	$(Sc)d/(S)e$ 1	5	8 (min after dilution)
^{14}C-Hydrocortisone	10^{-5}	2·20	0·11	0·09	0·08
^{14}C-Hydrocortisone	10^{-7}	2·86	0·20	0·12	ND
^{14}C-Hydrocortisone	10^{-9}	3·00	0·31	0·18	ND
^{3}H-Deoxycorticosterone	10^{-5}	7·80	1·22	1·00	0·98
^{3}H-Deoxycorticosterone	10^{-7}	8·21	1·35	1·08	ND
^{3}H-Deoxycorticosterone	10^{-9}	8·62	1·38	1·15	ND

Isolated peritoneal mast cells (V = 0·18) were incubated at 37° for 20 min in presence of ^{14}C-hydrocortisone or ^{3}H-deoxycorticosterone at the indicated concentrations. At the end of this incubation period, aliquots were removed and counted to give $(Sc)e/(S)e$ as described under "Materials and Methods". Cell suspensions were then diluted and sampled at the indicated time intervals to give $(Sc)d/(S)e$ as already described. Mean values from triplicate experiments are shown. $(S)e$ = moles of steroid per litre of medium; (Sc)e = moles of bound steroid per litre of packed cells, at equilibrium; $(Sc)d$ = moles of bound steroid per litre of packed cells, after dilution. $(Sc)e/(S)e$ corresponds to the zero time value of $(Sc)d/(S)e$. ND = not done.

Under conditions of equilibrium, saturation of membrane-binding sites would appear as a decrease in $(Sc)e/(S)e$ with increasing free steroid concentration in the medium $(S)e$. Since this decrease is slight with both ^{14}C-Hyc and ^{3}H-Doc, it seems reasonable to assume that binding of the two hormones to mast cells is highly dissociable and that membrane sites remain largely unsaturated.

By diluting (50-fold) equilibrated cell suspensions, most of bound ^{14}C-Hyc or ^{3}H-Doc dissociate in less than 1 min. Thus, at the higher steroid concentration (10^{-5} M), binding of ^{14}C-Hyc to mast cells decreases from a $(Sc)e/(S)e$ value of 2·20 before dilution (zero time) to 0·11 at 1 min after dilution; during the same time interval, the rate of ^{3}H-Doc binding goes from 7·80 to 1·22 (Table I). Such a rapid dissociation reflects nonspecific adsorption to cellular interfaces rather than specific binding to ,membrane receptors, and entirely accounts for the already noted

TABLE II.—*Effects of Hydrocortisone and Deoxycorticosterone on PGE_1-mediated Increase in Mast Cell Cyclic AMP*

Preincubation	Addition	cAMP (pmoles/10^6 mast cells)
Experiment I		
Medium	Medium	25·8±2·5
Medium	10 nM PGE_1	30·1±2·0
Medium	10 μM PGE_1	57·8±3·6*
Hydrocortisone	Medium	29·2±1·2
Hydrocortisone	10 nM PGE_1	43·6±1·8*
Hydrocortisone	10 μM PGE_1	70·2±5·3†
Experiment II		
Medium	Medium	18·6±1·1
Medium	10 nM PGE_1	24·3±2·2
Medium	10 μM PGE_1	36·8±2·7*
Deoxycorticosterone	Medium	15·7±0·8
Deoxycorticosterone	10 nM PGE_1	20·1±3·0
Deoxycorticosterone	10 μM PGE_1	31·7±2·8*

Isolated peritoneal mast cells (1×10^6 cells per ml) were incubated at 37° for 15 min with a balanced salt medium supplemented (or not) with 1 μM hydrocortisone or 1 μM deoxycorticosterone. At the end of this incubation period, the cells were challenged with medium or PGE_1 at the indicated concentrations and the incubation was prosecuted for an additional 15 min. Results are expressed as means ± s.e. mean (5 experiments). Differences from control values significant at: *$P < 0·05$; †$P < 0·01$.

absence of a direct correlation between equilibrium binding and anti-inflammatory activity.

Effects of hydrocortisone on PGE_1-mediated increase in cAMP and on 48/80-induced histamine release.

Once information on hydrocortisone binding to mast cells was available, it was of interest to ascertain whether such a binding, although highly dissociable, might still influence processes occurring at the interfaces thereby modifying the responsiveness of plasma membrane to stimuli supposed to be generated during the inflammatory response. For this purpose, isolated peritoneal mast cells (1×10^6 cells per ml) were incubated at 37° for 15 min in a medium supplemented with 1 μM hydrocortisone sodium succinate or 1 μM deoxycorticosterone. At the end of this incubation period, PGE_1 was added to each sample at the indicated concentrations and incubation was allowed to proceed for an additional 15 min before cooling, centrifuging and testing of cell pellets and supernatants for cAMP content. As shown in Table II, mast cells exposed to hydrocortisone undergo a greater increase in cAMP when subsequently challenged with PGE_1, as compared to untreated or deoxycorticosterone-treated cells. This enhancing effect is not simply caused by summation and, since it is still evident when PGE_1 is present at doses in the order of a few micrograms, it might prove to be pharmacologically relevant.

The hormone must continue to be present on the cell surface in order to sustain the response. Thus, mast cells preincubated with hydrocortisone, then exhaustively washed and resuspended in a steroid-free medium, turn out to be normally responsive to subsequent challenge with PGE_1 (results not shown). The slight increase in cAMP that occurs in cells exposed to hydrocortisone alone is probably due to some sort of nonspecific side-effect or to the release of trace amounts of prostaglandin by the same mast cells (Tolone *et al.*, 1978) or by contaminant leukocytes (Zurier and Sayadoff, 1975). Whatever the mechanism, the enhancing effect should be referred to the steroid component of the molecule, since free sodium succinate at equimolar concentrations is fully inactive.

As expected, increase in intracellular cAMP level prevents triggering of histamine release by Compound 48/80, an oligoamine which is a potent noncytotoxic stimulator of histamine discharge in mast cells (Paton, 1951). As shown in Table III, in cells exposed (at 37° for 15 min) to hydrocortisone or to PGE_1 alone and subsequently challenged with compound 48/80, histamine release mimics that of controls whereas, in cells simultaneously exposed to both hydrocortisone and PGE_1,

TABLE III.—*Effects of Hydrocortisone and PGE_1 on 48/80-induced Histamine Release from Peritoneal Mast Cells*

Preincubation	Addition	Histamine release (%)
Medium	Medium	$2 \cdot 0 \pm 0 \cdot 2$
Medium	48·80	$47 \cdot 6 \pm 4 \cdot 8$
Hydrocortisone	Medium	$1 \cdot 0 \pm 0 \cdot 1$
Hydrocortisone	48/80	$45 \cdot 0 \pm 3 \cdot 5$
PGE_1	Medium	$3 \cdot 0 \pm 0 \cdot 5$
PGE_1	48/80	$30 \cdot 0 \pm 4 \cdot 0$
Hydrocortisone + PGE_1	Medium	$3 \cdot 0 \pm 0 \cdot 8$
Hydrocortisone + PGE_1	48/80	$15 \cdot 0 \pm 2 \cdot 7$*

Isolated peritoneal mast cells (1×10^6 cells per ml) after having been preincubated at 37° for 15 min in a salt medium supplemented (or not) with 1 μM hydrocortisone, or 10 nM PGE_1, or both drugs, were challenged with medium or 1 μg/ml 48/80 in medium. Results are expressed as means ± s.e. mean (4 experiments).
*Differences from control values significant at $P < 0 \cdot 05$.

amine discharge is markedly reduced as compared to that of controls.

DISCUSSION

The results of this investigation demonstrate three important aspects of the interaction of hydrocortisone with rat peritoneal mast cells. First, the hormone can bind to mast cells under physiological conditions without inducing remarkable effects on intracellular cyclic AMP content or granule exocytosis. Second, this binding is highly dissociable, hence membrane sites remain largely unsaturated. Third, following steroid interaction mast cells become more susceptible to the action of PGE_1. As a consequence, cells undergo a greater increase in cAMP level, with the result that they are scarcely responsive at all to the histamine-releasing activity of Compound 48/80. Whatever the mechanism, this effect is a relatively specific one, since deoxycorticosterone cannot substitute for hydrocortisone in amplifying cyclase modulation.

If one considers that corticosteroids are hormones with a wide range of tissue specificity and that hydrocortisone exerts a regulatory influence on virtually every stage of the inflammatory process (Fauci, Dale and Balow, 1976), it is tempting to speculate that steroid-mediated membrane effects may provide a mechanism of considerable versatility through which tissue homoeostasis can be effected. Support for this view comes from the finding that hydrocortisone, by virtue of its membrane effects, markedly enhances Ca^{2+} binding to rat hepatocyte plasma membranes (Shlatz and Marinetti, 1972).

With regard to the inflammatory response, it has been demonstrated that prostaglandins of the E series act as extracellular regulators of cell function and that this type of modulation also applies to cells involved in secretion of inflammatory mediators (Bourne *et al.*, 1974; Morley, 1974). Therefore, it seems reasonable to assume that amplification of PGE_1 effects by hydrocortisone may contribute to its anti-inflammatory activity. The failure of deoxycorticosterone to synergize with PGE_1 correlates well with its inefficiency as an antiphlogistic agent and provides further support for this view.

Such a hormonal synergism might also account for other pharmacological effects of hydrocortisone, most notably inhibition of the reparative growth of fibroblasts. These cells do in fact synthesize and release E-type prostaglandins (Jaffe, Parker and Philpott, 1972) which, in their turn, regulate the growth of an unidentified fibroblast subpopulation (Ko, Page and Narayanan, 1977).

In considering equilibrium-binding data, it could be argued that features of hydrocortisone interaction with mast cells make it difficult to reconcile the nonspecific adsorption of steroid to the cell surface with the "synergism" hypothesis. However, there are some considerations which may help to resolve this issue. Under conditions where the interfacial regions between polar and non-polar media constitute a significant part of the total volume, a large fraction of any steroid hormone present is likely to be adsorbed at the interfaces at equilibrium, leaving only small amounts free in solution. In fact, at the lowest concentrations, at which physiological as well as pharmacological levels would presumably be approached, the ratio of adsorbed to free hormone would become greatest. Furthermore, the adsorbed hormone molecules would necessarily be brought into interaction with other compounds that are also adsorbed, and one might therefore expect that in some circumstances they would be able to take part in, or to influence, processes occurring at the interfaces. Synergism between hydrocortisone and PGE_1 might then be explained in terms of increased efficiency in PGE_1-receptor binding. Current evidence suggests that these receptors and adenylate cyclase are separate entities (Limbird and Lefkowitz, 1977) and that receptor interaction with cyclase is needed for their ensuing activation (Orly and Schramm, 1976). As stated by Malawista and co-workers (Rudolph,

Greengard and Malawista, 1977), the apparent affinity of this interaction is greatly enhanced when the receptor site is occupied by the appropriate effector. Thus, an increased efficiency in PGE_1-receptor-binding would lead to amplified prostaglandin-mediated cyclase activation.

Another possibility that should be considered is that hydrocortisone may influence the binding of Ca^{2+} to plasma membranes (Shlatz and Marinetti, 1972) or its influx inside the cell (Bangham, Standish and Weissmann, 1965). Also this effect might result in amplification since, as demonstrated by Sullivan *et al.* (1975*b*), Ca^{2+} exerts a negative feedback control on adenylate cyclase in purified mast cells.

Whatever the mechanism, the presence of polar groups at C11 or C17 of the sterol moiety appears to be essential for both anti-inflammatory and synergistic effects of corticosteroids. Further studies on isolated cell populations are necessary to clarify the functional significance of present data.

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