Structurally Based, Selective Interaction of Arsenite with Steroid Receptors*

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Steroid binding to cognate receptors is of high affinity. However, due to the appreciable homologies in the steroid-binding domains of receptors, this binding is hardly ever totally specific. We have recently obtained evidence that a vicinal dithiol group is involved in steroid binding to glucocorticoid receptors and that these vicinal dithiols are two of the three cysteines in the 16-kDa steroid-binding core. We now report that a comparison of the placement of cysteines in the comparable region of other receptors revealed a lack of similarly closely spaced thiols, which led to the prediction that arsenite would be totally selective in its interaction with glucocorticoid receptors. In fact, 100 μM arsenite inhibited all steroid binding to glucocorticoid receptors while having no effect on the binding of androgen, estrogen, mineralocorticoid, or progesterone receptors. Such total selectivity is not seen for selenite, which is another very potent inhibitor of glucocorticoid binding. This is the first report of absolute selectivity among steroid receptors that is based upon a known structural feature of the receptor protein. This selectivity of arsenite provides the easiest method to date for distinguishing between glucocorticoid and mineralocorticoid receptors and for selectively blocking steroid binding to glucocorticoid receptors in the assays of other receptors.

Steroid receptors exhibit both a common structural organization of functional domains and extensive homology between the steroid-binding domains (1). Thus, it is not surprising that virtually all steroids appear to interact with more than one class of receptors (2). It is possible to achieve total selectivity among the five classical steroid receptors (androgen, estrogen, mineralocorticoid, and progesterone) with anti-receptor antibodies. However, selective recognition of the biologically active form of receptors via the interaction with anti-receptor antibodies is based upon the tertiary structure of the receptor. Such a grouping of two thiols is not common in proteins (6) but has been found to be involved in steroid binding to glucocorticoid receptors (6, 7).

We now report that arsenite, but not selenite, recognizes a unique structural feature of glucocorticoid receptors. Thus arsenite specifically inhibited the binding to glucocorticoid, but not androgen, estrogen, mineralocorticoid, or progesterone receptors. This is the first report of simple, defined chemical interactions being able to distinguish among different steroid receptor proteins. These results offer a new approach for quantitating one receptor in a sample also containing glucocorticoid receptors and for distinguishing between mineralocorticoid and glucocorticoid receptors.

MATERIALS AND METHODS

Chemicals—[3H]Dexamethasone (Sigma) and [3H]dexamethasone (40 or 47 Ci/mmol, Amersham Corp.) were commercially available. TAPS (Ultrol grade) was purchased from Behring Diagnostics, HEPES was from Calbiochem, and sodium arsenite and sodium selenite pentahydrate were purchased from J. T. Baker Chemical Co. and Fluka, respectively. Methyl methanethiosulfonate (stored at 0 °C) was used as received from Aldrich. All [3H]-labeled samples were counted in Hydrofluor (National Diagnostics) at 40–55% counting efficiency in a Beckman 5801 liquid scintillation counter with automatic cpm-to-dpm conversion.

Buffers and Solutions—TAPS buffer (pH 8.8) was composed of 25 mM TAPS, 1 mM EDTA, and 10% glycerol; pH 8.2 TAPS buffer consisted of 10 mM TAPS, 1 mM EDTA, 50 mM NaCl, 20 mM NaMoO₄, and 10% glycerol. HEPES buffer (pH 7.5) contained 25 mM HEPES, 1 mM EDTA, and 20% glycerol. Each buffer was adjusted to the final pH at 0 °C with sodium hydroxide.

Cells and Preparation and Labeling of Receptors—MCF-7 cells (from Dr. Susan Bates, NIH) were grown at 37 °C under an atmosphere of 5% CO₂ in Richter's improved minimum essential medium supplemented with 10% fetal calf serum and 0.03% glutamine. GH₂ cells (from Dr. Bruce Weintaub, NIH) were grown in Swin's S77 medium supplemented with 5% fetal and 5% newborn bovine serum (Biofluids) and 0.03% glutamine as described for HTC cells (7). Cytosol was prepared from trypanized cells by freeze-thaw lysis (7)

The abbreviations used are: TAPS, 3-(N-tris(hydroxymethyl)amino)1-propanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid.
in pH 7.5 HEPES buffer and centrifugation at \( \geq 30,000 \times g \). Male Sprague-Dawley rats (3 days after adrenalectomy) were anesthetized and the kidneys perfused by aortic catheter with 70 ml of room temperature saline (first 6 ml containing 50 units/ml heparin). The cystosol was then prepared in pH 8.2 TAPS buffer by mechanical homogenization and centrifugation at \( \geq 100,000 \times g \) as described by Eisen and Harmon (10).

Cytosols containing the steroid-free receptors were used immediately or quickly thawed after storage in liquid N\(_2\). Diluted cytosols (30 or 60% (v/v) in buffer, rat kidney cytosol also contained 35% (v/v) of pH 8.8 TAPS buffer) to the pH and was preincubated with a 100-fold excess of RU 28,362 for 10 min) were labeled with \(^{3}H\)-steroid \( \pm \) excess \(^{3}H\)-steroid \( \pm 20 \) mM Na\(_2\)MoO\(_4\) (7). The \(^{3}H\)-steroids used for the various receptors (in parentheses) were: dexamethasone (glucocorticoid), 17\(\beta\)-estradiol (estrogen), R5020 + excess \(^{3}H\)dexamethasone (progesterone), 5a-dihydroxyetiocholanolone (androgon), and aldosterone + excess RU 28,362 (mineralocorticoid). Free

![FIG. 1. Homology of amino acid sequence around Cys-656 of the rat glucocorticoid receptor with other steroid receptors.](http://www.jbc.org/)

The sequence alignments given in Chang et al. (12) for human receptors were adapted to show the comparison with the 60 amino acids on either side of Cys-656 of the rat glucocorticoid receptor. The rat mineralocorticoid sequence was from Patel et al. (13). The difference between rat and human mineralocorticoid receptors is underlined. The solid boxes mark the positions of cysteine residues; "-" signifies the same amino acid as seen for the rat glucocorticoid receptor. "\(\times\)" indicates a gap that was introduced to maximize the alignment. The receptors listed are glucocorticoid (GR), progesterone (PR), mineralocorticoid (MR), androgen (AR), and estrogen (ER) from rat (r) or human (h) sources. The identity of amino acid 400 of the estrogen receptor was changed from Gly to Val to reflect a recently discovered cloning error (14).

![FIG. 2. Effect of arsenite on the steroid binding of human androgen, estrogen, glucocorticoid, progesterone, and rat mineralocorticoid receptors.](http://www.jbc.org/)

Steroid-free receptors in buffer containing 21 mM Na\(_2\)MoO\(_4\) were treated with various concentrations of sodium arsenite for 30 min and assayed for \(^{3}H\)-steroid binding; the average specific binding was then expressed as a percent of the untreated control for glucocorticoid (\(\bullet\)), estrogen (\(\Delta\)), progesterone (\(\triangle\)), androgen (\(\square\)), and mineralocorticoid (\(\bigcirc\)) receptors. Each point represents the average value from at least two experiments.

![FIG. 3. Effect of selenite on the steroid binding of human androgen, estrogen, glucocorticoid, progesterone, and rat mineralocorticoid receptors.](http://www.jbc.org/)

As for Fig. 2, steroid-free receptors were treated with various concentrations of sodium selenite in buffer for 30 min and assayed for \(^{3}H\)-steroid binding; the average specific binding was then expressed as a percent of the untreated control for glucocorticoid (\(\bullet\)), estrogen (\(\Delta\)), progesterone (\(\triangle\)), androgen (\(\square\)), and mineralocorticoid (\(\bigcirc\)) receptors. Each point represents the average value from at least two experiments.

Steroid was removed by adding a 10% dextran-coated charcoal suspension in buffer (added volume = 20% of reaction volume). Nonspecific binding labeling equaled that seen with excess competing \(^{3}H\)-steroid.

**RESULTS AND DISCUSSION**

We have recently found that steroid binding to the 16-kDa steroid-binding core fragment of the rat glucocorticoid receptor (11), which contains just the 3 cysteines Cys-640, -656, and -661, is inhibited by arsenite.\(^3\) Thus, two of these 3 cysteines are candidates for the vicinal diols involved in glucocorticoid binding.\(\dagger\) The common sensitivity of all steroid receptors to thiol reagents (3-5), and the identical organization of each steroid-binding domain, suggested that arsenite would be equally effective in blocking steroid binding to the other receptors. An examination of the steroid-binding domain of other receptors for homology around Cys-656 of the rat glucocorticoid receptor did reveal a common cysteine at 651 (rat glucocorticoid receptor numbering) for all but the estrogen receptor (Fig. 1). This cysteine, when oxidized or alkylated, may be responsible for the inhibition of steroid binding to these receptors. However, further examination of the region up to the carboxyl terminus of the 16-kDa core fragment at amino acid 673 (11) indicated that only the glucocorticoid receptor contained two closely spaced cysteines (Fig. 1). The three cysteines that are in this 16-kDa core fragment of the glucocorticoid receptor are 4 and 15 amino acids apart. In contrast, the closest cysteines in this region of the other receptors are 21 amino acids apart; they are 40 amino acids apart in the mineralocorticoid receptor. These structural features suggested that arsenite might inhibit steroid binding only to the glucocorticoid receptor. To test this hypothesis, we examined the binding of steroids to each of the other receptors. Extracts of MCF-7 cells were used as a source of human androgen, estrogen, glucocorticoid, and progesterone receptors; rat kidney was our source of mineralocorticoid receptors. We found that arsenite was equally effective in blocking all steroid binding to human (Fig. 2) and rat (6) glucocorticoid receptors at concentrations (100 \(\mu\)M) which caused no significant inhibition of binding to any of the other receptors.

steroid receptors (Fig. 2). This result also supports our conclusion that the vicinal dithiols involved in the binding to glucocorticoid receptors are both on the receptor itself and not on a common, associated nonreceptor molecule since arsenite does not block the binding of androgens, estrogens, or progesterins to their cognate receptors, which do contain hsp90 (15) and the 59-kDa protein (16) but not the appropriate closely spaced thiolos (see Fig. 1).

This specificity of arsenite for glucocorticoid receptors could reflect the fact that the inhibition of binding in Fig. 2 occurred at much lower concentrations of reagent than is seen for most other thiol reagents (4). For example, the affinity label dexamethasone 21-mesyIate (17) is active at 0.1 μM concentrations and covalently labels only glucocorticoid receptors (18). Since selenite inhibits glucocorticoid receptor binding (6, 8) at the same low concentrations as arsenite (6), we examined whether selenite was also selective for glucocorticoid receptors. The data of Fig. 3 show that selenite was as effective as arsenite in blocking glucocorticoid binding (cf. Fig. 2) but also inhibited the binding to other receptors. The 300 μM selenite that was required to block glucocorticoid binding also prevented binding to 35% of the progesterone receptors and 78% of the mineralocorticoid receptors. Thus not all thiol reagents that inhibit steroid binding to glucocorticoid receptors at low reagent concentrations are totally selective for glucocorticoid receptors.

We next investigated whether arsenite could replace 3H-glucocorticoids as a competitor of 3H-steroid binding to glucocorticoid receptors. In HTC cell cytosol, the amount of nonspecific binding by [3H]dexamethasone, as percent of total binding, was 3.9 ± 1.5% (S.D., n = 6) with a 500-fold excess of [3H]dexamethasone and 3.9 ± 1.2% (S.D., n = 6) with 100 μM arsenite. In the binding of [3H]R5020 to progesterone and glucocorticoid receptors in MCF-7 cytosol, 100 μM arsenite was just as effective as a 200-fold excess of [3H]dexamethasone in blocking R5020 binding to glucocorticoid receptors. Thus the total binding of [3H]R5020 in presence of 100 μM arsenite was 101 ± 7% (S.D., n = 4) of that seen in the presence of a 200-fold excess of [3H]dexamethasone. Similarly, the total binding of [3H]aldosterone in rat kidney cytosol in the presence of 100 μM arsenite was 107 ± 2% (n = 2) of that seen with a 120-fold excess of the “pure glucocorticoid” RU 28,362 (2, 10); the specific binding was somewhat higher (47 ± 19%, n = 2) in the presence of arsenite, presumably due to partial inhibition of [3H]aldosterone binding by the large excess of RU 28,362. Thus, arsenite offers a simple, straightforward method based on receptor structure (see Fig. 1) for discriminating between glucocorticoid and mineralocorticoid receptors, which can be notoriously difficult to accomplish (19–21).

Very few tissue culture cell lines are known to contain mineralocorticoid receptors. We have used the above specificity of arsenite inactivation of glucocorticoid receptors to investigate an earlier report that rat pituitary cells (GH1) contain mineralocorticoid receptors (22). We found that the presence of 30–1000 μM arsenite had no effect on the amount of [3H]aldosterone which specifically bound to GH1 cell cytosol in the presence of excess RU 28,362 (data not shown). This thus confirms that the specific binding of aldosterone in GH1 cell cytosol is to mineralocorticoid and not glucocorticoid receptors.

The unique interactions that occur between arsenite and the vicinal dithiols in the steroid-binding domain of glucocorticoid receptors to give the equivalent of a cyclic dithioarsenate adduct (6, 9) have numerous practical consequences. First, vicinal dithiols are relatively rare in proteins so that most proteins will be unaffected by arsenite (6). Second, arsenite can be used in place of more expensive steroids to compete for 3H-glucocorticoid binding in receptor assays. Arsenite may also provide a more accurate background value in some cases since the total amount of steroid present has not been increased by the addition of competing, nonradioactive steroid. Third, the specificity of arsenite for glucocorticoid receptors is greater than that of any steroid we know. This specificity of arsenite may prompt a new survey of mineralocorticoid receptors, which have been very difficult to distinguish from glucocorticoid receptors (19–21). Fourth, since there are all most no steroids that bind to only one class of receptors, it can be difficult to quantitate just one receptor (and block all of the cross-binding to other receptors) in the presence of multiple receptors, such as in mammary tissue which contains glucocorticoid, mineralocorticoid, and progesterone receptors (20, 23–26). The use of arsenite to selectively block glucocorticoid receptors (Fig. 2) may provide more accurate values for the progesterone receptor content of breast cancer tissues and thus improve the prognosticative value of these assays (27). Finally, the ready reversibility of arsenite inhibition by dithiothreitol (6) is unusual among selective inhibitors of steroid binding and should be useful in assay samples that are limited in quantity.

In conclusion, we have found that arsenite selectively inhibits steroid binding to glucocorticoid receptors (Fig. 2). Comparable specificity has not been observed previously for any steroid or chemical and is not seen for another potent thiol reagent, selenite (Fig. 3). The specificity depends on a vicinal dithiol grouping (6) that is unique to the glucocorticoid receptor (Fig. 1). This is the first time that all of the amino acid determinants for such specificity among steroid receptors have been determined. The apparent lack of these determinants in the retinoic acid (28), thyroid (29), and vitamin D3 (30) receptors suggests that arsenite will not affect the binding of these receptors.

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