Effect of Atrial Natriuretic Factor on Calcium Fluxes in Adrenal Glomerulosa Cells

MASAO TAKAGI, MARI TAKAGI, ROBERTO FRANCO-SAENZ, DAVID SHIER, AND PATRICK J. MULROW

SUMMARY We studied the effect of atrial natriuretic factor (ANF) on calcium influx and efflux in rat adrenal glomerulosa cells stimulated by angiotensin II (Ang II) or potassium ion, and observed how ANF inhibits the initial and sustained phases of the aldosterone response to Ang II or K+ using a superfusion system of dispersed adrenal glomerulosa cells. K+ (8 mM) significantly increased Ca2+ influx rate compared with basal rate (0.91 ± 0.10 vs 0.42 ± 0.04 nmol/min/106 cells; p<0.01). ANF (10^{-8} M) did not inhibit the K+-induced increase in Ca2+ influx rate (0.99 ± 0.18 nmol/min/106 cells). Ang II (10^{-9} and 10^{-8} M) stimulated Ca2+ influx rate (10^{-9} M Ang II, 0.62 ± 0.02; 10^{-8} M Ang II, 0.71 ± 0.09 vs basal, 0.44 ± 0.03 nmol/min/106 cells; p<0.05), while ANF (10^{-8} M) did not change the Ca2+ influx rate increased by Ang II (ANF + 10^{-8} M Ang II, 0.62 ± 0.06; ANF + 10^{-8} M Ang II, 0.69 ± 0.14 nmol/min/106 cells). In the Ca2+ efflux study ANF (10^{-8} M) was perfused through the cells 10 minutes before the start of perfusion with Ang II (10^{-9} M) or K+ (12 mM). The peak Ca2+ fractional efflux ratio elicited by Ang II or K+ did not change in the presence of ANF (K+, 1.374 ± 0.051 vs K+ + ANF, 1.388 ± 0.011; Ang II, 1.432 ± 0.066 vs Ang II + ANF, 1.394 ± 0.017). The stimulatory effects of Ang II and K+ and the inhibitory effect of ANF on aldosterone production were more potent in the superfusion system of dispersed adrenal glomerulosa cells than in the static incubation system. ANF inhibited aldosterone production in both the initial phase and the sustained phase. These results suggest that ANF does not affect Ca2+ influx or efflux in rat adrenal glomerulosa cells and that the inhibitory effect of ANF must occur after the generation of the Ca2+ signal in the cells. (Hypertension 11: 433-439, 1988)

KEY WORDS • atrial natriuretic factor • aldosterone • calcium influx • calcium efflux

Atrial natriuretic factor (ANF) inhibits aldosterone production in adrenal glands in vitro and in vivo.1-5 Infusion of ANF lowers plasma aldosterone levels in humans.6,7 ANF also has smooth muscle-relaxing activities.8,9 The intracellular mechanisms mediating the actions of ANF are still unknown. The calcium messenger system mediates the cellular response to some regulators in these two target cells for ANF, namely, adrenal glomerulosa cells and vascular smooth muscle cells.10 We examined whether ANF affects the calcium messenger system in adrenal glomerulosa cells. The response of aldosterone production in adrenal glomerulosa cells has two phases (i.e., initial and sustained). The mechanism by which angiotensin II (Ang II) stimulates the two phases differs from those of potassium ion and adrenocorticotropic hormone (ACTH).11 However, these three secretagogues increase Ca2+ influx and efflux in the glomerulosa cells.11-13 In the present study, we evaluated the effect of ANF on Ca2+ influx and efflux in rat adrenal glomerulosa cells stimulated by Ang II or K+ and observed the time course of the inhibition of the two phases of response, using a superfusion system of adrenal glomerulosa cells.

Materials and Methods

Synthetic rat ANF (28 amino acids) was purchased from Peninsula Laboratories (Belmont, CA, USA). Synthetic [Asp, Ile]Ang II and bovine serum albumin were obtained from Sigma Chemical (St. Louis, MO, USA), collagenase from Worthington Diagnostics Division (Millipore, Freehold, NJ, USA), 45CaCl2 from Amersham (Arlington Height, IL, USA), Medium 199 with Hanks' salts from Gibco (Grand Island, NY, USA), and aldosterone radioimmunoassay kit from Diagnostic Products (Los Angeles, CA, USA).
Preparation of Adrenal Glomerulosa Cells

Female Sprague-Dawley rats (weight, 180–220 g; Harlan, Indianapolis, IN, USA) maintained on a regular sodium diet (Wayne Lab Blox, Allied Mills, Chicago, IL, USA) were used for the experiments. The rats were killed by decapitation, and the capsular portions of the adrenal glands were separated by incising the capsule and squeezing the decapsular portions. Minced capsular tissues were incubated with collagenase (2 mg/ml) in Medium 199 with Hanks’ salts for 30 minutes at 37°C under an O₂ atmosphere and dispersed by repeated agitation using a pipette. Medium 199 with Hanks’ salts contained 1.25 mM Ca²⁺, 3.5 mM K⁺, 5.5 mM glucose, and 0.2% bovine serum albumin and was adjusted to pH 7.4. The cell suspensions were filtered through a nylon mesh (70 μm pore size) and centrifuged at 100 g for 15 minutes. The cell pellets were resuspended in fresh Medium 199 and used for the Ca²⁺ influx study and the superfusion study.

For the Ca²⁺ influx study, the cells were loaded onto a 0 to 60% linear gradient of colloidal silica (Percoll, Pharmacia, Piscataway, NJ, USA) solution in Medium 199 and centrifuged at 800 g for 15 minutes to remove contaminating red blood cells, fasciculata cells, and damaged cells. A band containing glomerulosa cells was obtained above a band of red blood cells and beneath the layers of fasciculata cells and of damaged cells or cell debris.

The separated glomerulosa cells were filtered through nylon mesh to remove lipid lumps produced by the cells. After three washings, the cells were reconstituted with fresh media containing 2% bovine serum albumin for the Ca²⁺ influx study. These glomerulosa cell suspensions were contaminated with 1 to 3% fasciculata cells, but were almost free of red blood cells and damaged cells. For the Ca²⁺ influx study with K⁺ stimulation, Krebs-Ringer bicarbonate glucose buffer (KRBGA), in the same concentrations of Ca²⁺, K⁺, glucose, and bovine serum albumin, was used. In this case all incubations were done under 95% O₂, 5% CO₂ to maintain pH 7.4. The basal Ca²⁺ influx rate and the K⁺-stimulated influx rate obtained with KRBGA were not different from those obtained with Medium 199 (as described in Results).

Ca²⁺ Influx Study

The Ca²⁺ influx was measured according to the method previously described by Mauger et al. and Kojima et al. In brief, incubation was started by adding 500 μl of cell suspension (1.5–3 × 10⁶ cells/ml) to a tube containing 5 μCi of ⁴⁵CaCl₂, Ang II (10⁻⁹ or 10⁻⁸ M) or KC1 (8 mM), or ANF (10⁻⁸ M) in a volume of 10 μl at 37°C under O₂. The ⁴⁵Ca²⁺ uptake was determined by taking 100-μl samples at 15, 45, 75, and 105 seconds. Each sample was immediately diluted in 4 ml of ice-cold washing solution containing 144 mM NaCl, 5 mM CaCl₂, and 5 mM Tris HCl (pH 7.4). The mixture was filtered through a Whatman GF/C glass fiber filter (Clifton, NJ, USA) and washed with 50 ml of the ice-cold washing solution. The radioactivity associated with the filter was counted with 84% counting efficiency. Background counts were determined by washing 100 μl of media containing ⁴⁵Ca²⁺ (1 μCi) without cells on the filter in each experiment. ⁴⁵Ca²⁺ uptake at 15 seconds was always higher in K+-stimulated or Ang II–stimulated groups than in the control (basal), and extrapolation of linear regression lines of ⁴⁵Ca²⁺ uptake to the ordinate also gave higher values in K⁺-stimulated or Ang II–stimulated groups. This result suggested that the increase in Ca²⁺ uptake elicited by K⁺ or Ang II occurred more rapidly than expected.

The Ca²⁺ influx rate was calculated by subtracting the background counts instead of the extrapolated values from ⁴⁵Ca²⁺ uptake at 60 seconds obtained from the regression line. In additional experiments, we checked the effect of a calcium channel blocker, verapamil (2 × 10⁻³ M), on Ca²⁺ influx stimulated by K⁺.

Ca²⁺ Efflux Study

The Ca²⁺ efflux study was done according to the method of Kojima et al. In short, dispersed adrenal capsule cells suspended in 2 ml of Medium 199 were prelabeled by incubation with ⁴⁵CaCl₂ (10 μCi/ml) at 37°C under O₂ for 60 minutes. The suspension was filtered through 70-μm nylon mesh and centrifuged at 100 g for 10 minutes. The cell pellet was resuspended in fresh media, and 500 μl of cell suspension (3–4 × 10⁶ cells/ml) was applied to a column containing 0.5 ml of Sephadex G-15. The column was placed in a water bath at 37°C and perfused at a flow rate of 0.5 ml of media per minute. Two-minute (1 ml) fractions were collected for the measurement of ⁴⁵Ca²⁺. After 30 minutes of basal perfusion, the cells were superfused first with media containing 10⁻⁴ M ANF for 10 minutes and then with ANF plus 10⁻⁸ M Ang II or 12 mM K⁺ in media for 30 minutes. At the end of the experiment (i.e., 120 minutes after the start of superfusion), perchloric acid (0.6 M) was pumped for 30 minutes through the column to lyse the cells and measure ⁴⁵Ca²⁺ remaining in the cells. The radioactivity fractional efflux was calculated from the radioactivity released per unit time (2 minutes) and was expressed as a percentage of the mean cell radioactivity in the cells during the collection period. The fractional efflux ratio (FER) is the fractional efflux of ⁴⁵Ca²⁺ released from the stimulated cells divided by the fractional efflux of ⁴⁵Ca²⁺ from unstimulated controls. In some experiments, verapamil (2 × 10⁻⁵ M) instead of ANF was superfused to check the effect of the calcium channel blocker on Ca²⁺ efflux stimulated by K⁺. The dead space of the system was approximately 2 ml.

Aldosterone Production in the Superfusion System of Dispersed Adrenal Glomerulosa Cells

The same procedures were followed as described for the Ca²⁺ efflux study, except that 500 μl of cell suspension (1–1.5 × 10⁶ cells/ml) was applied to the column without ⁴⁵Ca²⁺ prelabeling and that 4-minute fractions (1 ml each) were collected at a flow rate of 0.25 ml/min after a 30-minute static incubation and a 30-minute washout perfusion. To observe the effect of
ANF on the initial phase of aldosterone response to Ang II or K⁺, ANF was added to the cells 10 minutes before the start of the stimulation of Ang II (10⁻⁹ M) or K⁺ (12 mM) and the infusion was continued during the 30-minute stimulation. In addition, ANF was added 20 minutes after the start of the 50-minute stimulation and continued for 30 minutes to see the effect of ANF on the sustained phase of the response. Aldosterone in each fraction of perfusate was measured without extraction using the radioimmunoassay kit.

**Statistical Evaluation**

Results are shown as the means ± SE. Statistical analysis was done with analysis of variance, the multiple comparison method by Dunnett,¹⁶ and the Tukey-Kramer method.

### Results

**Effect of ANF on Ca²⁺ Influx Stimulated by K⁺ or Ang II**

Figure 1 shows ⁴⁵Ca²⁺ uptake into purified rat adrenal glomerulosa cells. ⁴⁵Ca²⁺ uptake was significantly stimulated by the increase in K⁺ concentration (from 3.5 to 8 mM) or Ang II (10⁻⁸ M) as compared with basal uptake at every sampling time (15–105 seconds). However, the stimulation of Ca²⁺ uptake was not inhibited by ANF (10⁻⁸ M) at any sampling time. The Ca²⁺ influx rate was calculated from the regression lines obtained in each experiment (Tables 1 and 2).

Table 1 shows the effect of ANF and verapamil on the Ca²⁺ influx rate in adrenal glomerulosa cells stimulated by K⁺ using KRBGA buffer. K⁺ significantly stimulated the Ca²⁺ influx rate (0.91 ± 0.10 vs 0.42 ± 0.04 nmol/min/10⁶ cells; p<0.01).

ANF did not block the Ca²⁺ influx response to K⁺ (0.99 ± 0.18 nmol/min/10⁶ cells), while verapamil blocked the Ca²⁺ influx stimulated by K⁺ (0.50 ± 0.12 nmol/min/10⁶ cells). Our preliminary experiments showed that the aldosterone response to Ang II stimulation is better in Medium 199 than in KRBGA buffer. Therefore, we used Medium 199 instead of KRBGA buffer to evaluate the effect of ANF on Ca²⁺ influx stimulated by Ang II.

Basal and K⁺-stimulated Ca²⁺ influx rates in Medium 199 were the same as in KRBGA buffer (see Table 2). As shown in Table 2, two different concentrations of Ang II (10⁻⁹ and 10⁻⁸ M) significantly stimulated Ca²⁺ influx (0.62 ± 0.02 and 0.71 ± 0.09, respectively, vs 0.44 ± 0.03 nmol/min/10⁶ cells; p<0.05). However, the presence of ANF (10⁻⁸ M) did not change Ca²⁺ influx stimulated by the two concentrations of Ang II (10⁻⁹ M Ang II + ANF, 0.62 ± 0.06; 10⁻⁸ M Ang II + ANF, 0.69 ± 0.14 nmol/min/10⁶ cells).

### Effect of ANF on Ca²⁺ Efflux Stimulated by K⁺ or Ang II

Figure 2 shows ⁴³Ca²⁺ release from ⁴³Ca²⁺-preloaded cells into each fraction of perfusate during K⁺ stimulation in a typical experiment. An increase in K⁺ in the media (3.5 to 12 mM) elicited Ca²⁺ release from the cells. In two pilot experiments, verapamil (2 x 10⁻⁵ M) reduced the Ca²⁺ efflux in response to K⁺ by 70%, whereas ANF (10⁻⁸ M) did not change it. The rate of Ca²⁺ efflux expressed as FER was in-

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**Table 1. Effect of ANF on K⁺-Stimulated Ca²⁺ Influx Rate**

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>No. of samples</th>
<th>Ca²⁺ influx (nmol/min/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>24</td>
<td>0.42 ± 0.04</td>
</tr>
<tr>
<td>K⁺ (8 mM)</td>
<td>11</td>
<td>0.91 ± 0.10*</td>
</tr>
<tr>
<td>K⁺ (8 mM) + ANF (10⁻⁸ M)</td>
<td>5</td>
<td>0.99 ± 0.18*</td>
</tr>
<tr>
<td>K⁺ (8 mM) + verapamil (2 x 10⁻⁵ M)</td>
<td>2</td>
<td>0.50 ± 0.12</td>
</tr>
</tbody>
</table>

Values represent means ± SE.

Ca²⁺ influx was measured using Krebs-Ringer buffer as described in Materials and Methods.

* p<0.01, compared with basal value.
Table 2. Effect of ANF on Ang II-stimulated Ca2+ influx rate

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>No. of samples</th>
<th>Ca2+ influx (nmol/min/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>7</td>
<td>0.44±0.03</td>
</tr>
<tr>
<td>Ang II (10⁻⁹ M)</td>
<td>4</td>
<td>0.62±0.02*</td>
</tr>
<tr>
<td>Ang II (10⁻⁹ M)+ANF (10⁻⁸ M)</td>
<td>4</td>
<td>0.62±0.06*</td>
</tr>
<tr>
<td>Ang II (10⁻⁸ M)</td>
<td>5</td>
<td>0.71±0.09*</td>
</tr>
<tr>
<td>Ang II (10⁻⁸ M)+ANF (10⁻⁸ M)</td>
<td>2</td>
<td>0.69±0.14</td>
</tr>
<tr>
<td>K⁺ (8 mM)</td>
<td>3</td>
<td>0.91±0.11*</td>
</tr>
</tbody>
</table>

Values represent means±SE. Ca2+ influx was measured using Medium 199 as described in Materials and Methods.

*p<0.05, compared with basal value.

Increased by adding K⁺, but it was not modified in the presence of ANF, as shown in Figure 3 (top panel; peak of FER: K⁺ alone, 1.374±0.051; K⁺+ANF, 1.388±0.011). Figure 3 (bottom panel) demonstrates that ANF did not reduce the increase in FER elicited by Ang II (peak of FER: Ang II alone, 1.432±0.066; Ang II+ANF, 1.394±0.017). Both figures show that ANF did not have any significant effect on basal Ca2+ efflux before the stimulants were added.

Aldosterone Production in the Superfusion System of Dispersed Adrenal Glomerulosa Cells

Figures 4 and 5 demonstrate the time course of aldosterone release from the dispersed adrenal glomerulosa cells in each column. Both 12 mM K⁺ and 10⁻⁹ M Ang II markedly stimulated aldosterone production. ANF (10⁻⁸ M), which had been added 10 minutes beforehand, inhibited the initial phase of the aldosterone response (see Figure 4). The values of aldosterone production after stimulation by K⁺ or Ang II in the presence or absence of ANF are shown in Table 3.

Figure 5 demonstrates that the addition of ANF 20 minutes after the start of the stimulation with either K⁺ or Ang II caused a rapid fall in the sustained phase of aldosterone production, indicating that ANF inhibits both phases of the aldosterone response.

Discussion

ANF inhibits aldosterone production in adrenal glomerulosa cells stimulated by the three major regulators (i.e., Ang II, K⁺, and ACTH) in vitro. ANF also inhibits the increase in plasma aldosterone level in response to the administration of Ang II or KCl in vivo. Recent studies have shown that the three major regulators of aldosterone production act on adrenal cells through the intracellular calcium messenger system. In the case of Ang II stimulation, initial major changes are an increase of Ca2+ influx across plasma membrane and hydrolysis of phosphatidylinositol 4,5-bisphosphate, which gives rise to diacylglycerol and inositol 1,4,5-triphosphate. Inositol 1,4,5-triphosphate stimulates the release of Ca2+ from an intracellular store, causing a transient rise in the Ca2+ concentration in the cell cytosol. This rise in the cytosolic Ca2+ leads to the activation of calmodulin-dependent processes responsible for the initiation of the aldosterone secretory response. One of the calcium-dependent enzymes that is activated by the cytosolic Ca2+ is the plasma membrane calcium pump. Its activation causes an increase in the rate of Ca2+ efflux from the cells. The rise in diacylglycerol content of the plasma membrane, along with the transient rise in the
Ca\textsuperscript{2+} concentration, brings about the activation of Ca\textsuperscript{2+}-sensitive state and regulates the sustained phase of cellular response. In the case of K\textsuperscript{+} or ACTH stimulation, an increase in Ca\textsuperscript{2+} influx and in cyclic adenosine 3',5'-monophosphate (cAMP) content are the initial major changes. These activate calmodulin-dependent enzymes and A-kinase, respectively. A-kinase is the enzyme responsible for the sustained phase of the response. The difference between the actions of K\textsuperscript{+} and ACTH is thought to be in the relative magnitude of the two messengers, Ca\textsuperscript{2+} influx being the predominant one with K\textsuperscript{+} and cAMP with ACTH. One group reported that ANF did not alter the inhibitory effect of Ang II on Ca\textsuperscript{2+} fluxes in bovine glomerulosa cells measuring \textsuperscript{45}Ca\textsuperscript{2+} uptake during the first 15 minutes.'\textsuperscript{19} In their experimental system Ang II exerted an inhibitory effect on Ca\textsuperscript{2+} fluxes instead of a stimulatory effect. They did not study Ca\textsuperscript{2+} influx and efflux separately in the early phase of the response. In the present investigation, we studied separately the effect of ANF on Ca\textsuperscript{2+} influx and efflux in rat glomerulosa cells after Ang II and K\textsuperscript{+} stimulation.

The superfusion system of dispersed glomerulosa cells demonstrated that the aldosterone response to Ang II, K\textsuperscript{+}, or ANF is greater than in the static incubation system (see Table 3). For example, the increase in aldosterone production in response to Ang II was approximately 100-fold, while it is only twofold to sixfold in the static incubation system.\textsuperscript{1,4} The difference

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>Basal 56–60 min</th>
<th>Ang II 60–64 min</th>
<th>Ang II + ANF 64–68 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>12.9 ± 1.8</td>
<td>10.9 ± 1.5</td>
<td>11.1 ± 1.2</td>
</tr>
<tr>
<td>Ang II</td>
<td>1145.0 ± 68.0*</td>
<td>1046.9 ± 81.5*</td>
<td>1007.4 ± 121.0*</td>
</tr>
<tr>
<td>Ang II + ANF</td>
<td>121.4 ± 4.3†</td>
<td>121.3 ± 9.0†</td>
<td>106.9 ± 13.0†</td>
</tr>
<tr>
<td>K\textsuperscript{+}</td>
<td>1790.2 ± 152.5*</td>
<td>1717.9 ± 91.1*</td>
<td>1719.1 ± 102.5*</td>
</tr>
<tr>
<td>K\textsuperscript{+} + ANF</td>
<td>391.8 ± 42.3‡</td>
<td>376.9 ± 47.9‡</td>
<td>352.1 ± 25.0‡</td>
</tr>
</tbody>
</table>

Values show means ± SE in three consecutive fractions during 56 to 68 minutes of three experiments.
may be explained by the fact that in the superfusion system fresh media containing Ang II is always being supplied to cells; Ang II is easily destroyed during incubation with cells. Another possibility is that toxic metabolites from the cells and breakdown products in the media that might interfere with the cellular metabolism are continuously washed out. As shown in Figures 4 and 5, the aldosterone response occurred immediately after the administration of Ang II or K+, and the high output was maintained during the stimulation. ANF inhibited both the initial and the sustained phases of the response.

In the Ca2+ influx study, two different concentrations of Ang II (10^-9 and 10^-8 M) and 8 mM K+ significantly increased Ca2+ influx. Verapamil (2 x 10^-5 M) blocked the increase in Ca2+ influx stimulated by K+, which demonstrates that our experimental system could detect the specific effect of the Ca2+ channel blocker. In this system, ANF did not alter the Ca2+ influx rate stimulated by K+ or Ang II. These findings conflict with the result reported recently by Chartier and Schiffrin.20 They reported that Ca2+ influx stimulated by Ang II, ACTH, K+, or the dihydro-xyridine Bay k8644 was decreased by ANF (10^-5 M), using rat adrenal capsular cells. In our experiments the adrenal capsular cell suspension contained a large number of red blood cells, damaged cells, and cell debris, so in order to purify the glomerulosa cells and remove potential interference by the other cells it was necessary to use a Percoll gradient, as described by Kojima et al.12 Chartier and Schiffrin20 did not purify their adrenal capsular cell suspension, and that may have led to nonspecific effects caused by the other cells or damaged cells. In our experiment the basal influx rate was 0.44 ± 0.03 nmol/min/10^6 cells in Medium 199. These values are not different from those (0.39–0.75 nmol/min/10^6 cells) of Mauger et al.13 using rat liver cells, as calculated from their results, but they are one order of magnitude higher than those reported by Chartier and Schiffrin (0.053–0.056 nmol/min/10^6 cells).20

In the Ca2+ efflux study, the FER stimulated by K+ was similar to the FER (1.5) in the experiment performed by Kojima et al.13 using bovine adrenal cells. On the other hand, the increase in FER caused by Ang II was lower than in their data (3.5-fold). This discrepancy could be attributed to the species differences as reported by Kojima et al.12 Chartier and Schiffrin20 did not purify their adrenal capsular cell suspension, and that may have led to nonspecific effects caused by the other cells or damaged cells. In our experiment the basal influx rate was 0.44 ± 0.03 nmol/min/10^6 cells in Medium 199. These values are not different from those (0.39–0.75 nmol/min/10^6 cells) of Mauger et al.13 using rat liver cells, as calculated from their results, but they are one order of magnitude higher than those reported by Chartier and Schiffrin (0.053–0.056 nmol/min/10^6 cells).20

In conclusion, our results indicate that ANF does not affect Ca2+ influx or efflux in rat adrenal glomerulosa cells and that the inhibitory effect of ANF must occur after the generation of the Ca2+ signal in the cells.

References
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M Takagi, M Takagi, R Franco-Saenz, D Shier and P J Mulrow

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