Insulin-dependent contractility of glomerular mesangial cells in response to angiotensin II, platelet-activating factor and endothelin is attenuated by prostaglandin E₂

Marjorie E. DUNLOP and Richard G. LARKINS

University of Melbourne, Department of Medicine, Royal Melbourne Hospital, Parkville, Victoria 3050, Australia

Culture of glomerular mesangial cells in the absence of insulin decreased the degree of contraction of individual cells in response to vasoconstrictive agonists, angiotensin II, platelet-activating factor and endothelin 1, as compared with cells cultured in the presence of insulin (0.7 nM). This change was associated with a decreased sensitivity of the intracellular Ca²⁺ response to vasoactive agents in fura-2-loaded cells and with an increase in the basal level of prostanoid [prostaglandins (PG) E₁ and E₂] production estimated by radioimmunoassay. Addition of exogenous PGE₂ to insulin-exposed cells decreased the contractile response to that observed in insulin-deficient cells. Inclusion of 8-bromo cyclic AMP had a similar effect. In ⁴⁵Ca²⁺-release studies it was shown that, in saponin-permeabilized insulin-exposed cells, preincubation with exogenous PGE₂ or 8-bromo cyclic AMP decreased the sensitivity of ⁴⁵Ca²⁺ release in response to Ins(1,4,5)P₃, as demonstrated by an increase in the EC₅₀ (concn. giving half-maximal effect) to 0.182 ± 0.024 μM and 0.457 ± 0.031 μM respectively, as compared with untreated permeabilized cells (EC₅₀ 0.091 ± 0.021 μM). A similar decrease in Ins(1,4,5)P₃-sensitive ⁴⁵Ca²⁺ release was seen in permeabilized cells from insulin-free conditions of culture (EC₅₀ 0.20 ± 0.061 μM). As altered glomerular haemodynamics are found in insulinopaenic diabetic conditions, it is proposed that a decrease in intracellular Ca²⁺ availability in response to vasoactive agonists and consequent decrease in mesangial-cell contractility contributes to the hyperfiltration seen in this condition.

INTRODUCTION

The contractile response of glomerular mesangial cells to circulating or intra-glomerular factors is believed to contribute significantly to glomerular haemodynamics by regulation of capillary surface area and thus the ultrafiltrational coefficient (Ichikawa *et al.*, 1979; Dworkin *et al.*, 1983; reviewed by Mené *et al.*, 1989). As the mesangial cell has many of the properties of a modified smooth-muscle cell, it is expected that Ca²⁺ activation of calmodulin-dependent myosin light-chain kinase with subsequent phosphorylation of myosin light chains and stimulation of actin–myosin interactions, as in smooth muscle (reviewed by Kamm & Stull, 1985), is responsible for the shape change seen in isolated mesangial cells after stimulation with agonists which increase intracellular Ca²⁺ ([Ca²⁺]_i). Early insulin-dependent diabetes is characterized by hyperfiltration (Mogensen & Andersen, 1975) and there is considerable evidence from diabetic models that increased vasodilatory prostaglandins may contribute to this hyperfiltration (Craven *et al.*, 1987). Vasodilatation in response to prostaglandins may be effected via phosphorylation of myosin light-chain kinase by cyclic-AMP-dependent protein kinase, decreasing its affinity for calmodulin (Adelstein *et al.*, 1978), and thus the level of the holoenzyme, and accelerating the process of relaxation. Alternatively, in diabetes contractile responses may be decreased after the decrease in membrane phosphoinositides from which Ca²⁺-mobilizing InsP₃ is derived. Raised cyclic AMP levels may amplify this defect through cyclic AMP-dependent phosphorylation and alteration of Ca²⁺-mobilizing receptors (Supattapone *et al.*, 1988) and thus modification of agonist-induced contractility. Contractile agonists cause an elevation of [Ca²⁺]_i derived from both intra- and extra-cellular sources in cultured mesangial cells (Mené *et al.*, 1989). Mesangial cells in culture have been shown to have an absolute requirement

for insulin in order to contract in response to angiotensin II (AII) (Kriesberg, 1982). However, in contrast with this early description, sustained glomerular contractile potential has been recently demonstrated in two rat models of diabetes, one streptozotocin-induced and thus insulinopaenic, and the other genetic (Barnett *et al.*, 1987). The present study examines the insulin requirement for cultured mesangial cells to respond to potential contractile agents (AII), platelet-activating factor (PAF) and endothelin 1 (ET1) and the associated levels of [Ca²⁺]_i and prostanoid production.

The role of prostaglandins in inhibiting the contractile response was also investigated. In order to determine whether failure of mobilization of intracellular Ca²⁺ stores may have contributed to impaired contractile responses, the effect of exogenous Ins(1,4,5)P₃ on the release of ⁴⁵Ca²⁺ in permeabilized cells was studied.

MATERIALS AND METHODS

Culture of mesangial cells

Isolated glomeruli were prepared from the kidney cortices of male Sprague–Dawley rats by differential sieving and centrifugation by a published method (Hassid *et al.*, 1986). The glomeruli were further digested with collagenase to obtain mesangial-cell-enriched glomerular cores, from which mesangial cells were grown. Cells were maintained in medium RPMI-1640 supplemented with 15% (v/v) fetal-bovine serum, insulin (7 μM), penicillin (100 units/ml) and streptomycin (100 μg/ml). After 7–10 days these cells were separated from any remaining glomerular core, subcultured once in the above medium, and a preparation of pure mesangial cells was obtained by limiting-dilution techniques. Cells were then used after 5–10 passages,

Abbreviations used: [Ca²⁺]_i, intracellular Ca²⁺ concentration; AII, angiotensin II; PAF, platelet-activating factor; ET1, endothelin 1; PGE₂, prostaglandin E₂; 8BrCAMP, 8-bromo cyclic AMP; BAPTA, bis-(*O*-aminophenoxy)ethane-*NNN'*-tetra-acetate; EC₅₀, concn. giving half-maximal effect.

where they maintained a typical spindle-shaped smooth-muscle-cell-like appearance, and were shown to contain extensive microfilaments on transmission electron microscopy and to be susceptible in culture to the addition of mitomycin C. Cells were subcultured on to individual glass coverslips (3.5 cm²) or into 9.62 cm² culture dishes (Flow Laboratories). The medium was replaced by Dulbecco's Modified Eagle's Medium, containing 10% fetal-bovine serum with or without added insulin at concentrations of 7 μ M or 0.7 nM without antibiotics, during this 1–2-day culture period and through all subsequent subcultures. In the absence of added insulin, the insulin concentration of the medium was shown to be < 3.5 pM. When present, indomethacin (1 μ M) was added to subcultured cells for 18 h before experimentation.

Determination of [Ca²⁺]_i

Changes in cytosolic Ca²⁺ in mesangial-cell monolayers were determined after cell loading with the acetomethoxy ester (AM) of fura-2 (4–10 μ M). The Ca²⁺-associated fura-2 fluorescence was measured at excitation wavelengths of 340 and 380 nm, with an emission wavelength of 505 nm in a SPEX CM-2 cation-measuring system, at 37 °C in a cuvette containing 2 ml of Krebs–Ringer bicarbonate buffer (KRBB). [Ca²⁺]_i was quantified, after addition of AII (1 nM–1 μ M), PAF (0.1 nM–0.1 μ M), ET1 (1 nM–0.1 μ M) or BAY K 8644 (1 μ M–0.1 mM) in cell monolayers which had been cultured at the above insulin concentration and preincubated in buffer (KRBB) alone or containing EGTA (4 mM), 8-bromo cyclic AMP (8Br-cAMP) (1 nM–1 μ M) or the acetoxymethyl ester of bis-(*O*-aminophenoxy)ethane-*NNN'*-tetra-acetate (BAPTA). Free Ca²⁺ concentration was calculated with the formula $K_d[(F - F_{min.}) / (F_{max.} - F)]$, with $F_{max.}$ and $F_{min.}$ obtained after cell solubilization and the application of 10 μ M of the non-fluorescent ionophore ionomycin with CaCl₂ (10 mM) and MnCl₂ (1 mM) respectively and $K_d = 224$ nM as described previously (Dunlop & Larkins, 1989).

Mesangial-cell contractility

Video-microscopy was used to obtain real-time estimations of mesangial-cell length from microscopic fields chosen to display discernible cell boundaries, seen in sub-confluent cultures grown on glass coverslips. The cells were observed with a Leitz Dialux Microscope coupled to a RCA TC 1005/01 video-camera and a high-resolution black-and-white television monitor. Images were analysed directly at the monitor by using electronic video-calipers which generated two cursor lines on the monitor, which could be separated by providing a signal proportional to the cell dimension being measured (Hill & Larkins, 1989). After calibration by repeated measurement of a lined graticule, cell length was displayed on a digital panel meter and recorded as μ m. Cells were maintained in KRBB on a heated stage. Where appropriate, short preincubations were performed at the microscope and incubation conditions were identical with those employed for [Ca²⁺]_i.

ATP-dependent Ca²⁺ uptake and release from permeabilized cells

Cells were suspended in an intracellular medium with 0.2 mM-ATP and calculated free Ca²⁺ 150 nM as described previously (Dunlop & Larkins, 1988) and permeabilized with saponin (0.005%) for 10 min at 37 °C, followed by several washings in saponin-free medium. Incubations were in a final volume of 500 μ l containing 10⁵ cells and ⁴⁵Ca (1 μ Ci/ml).

Incubations were terminated by rapid (1 s) centrifugation in a Beckman Microfuge. Cells were washed twice in the ionic medium without added ATP, and the ⁴⁵Ca content was determined by

liquid-scintillation counting of a gel made with water/pseudocumene scintillation cocktail (2:5, v/v). When present, heparin (4–6 kDa) was added during the uptake period (Ghosh *et al.*, 1988). Rapid addition of Ins (1,4,5)P₃, was made at 5 min, and incubation was continued for a further 5–300 s before termination as above.

Prostaglandin E (PGE) radioimmunoassay

Total prostanoid PGE activity was measured by specific radioimmunoassay. [5,6,8,11,12,14,15(n)-H]PGE₂ (sp. radioactivity 140–107 Ci/mol) was used as tracer, and an anti-PGE₂ antibody with 100% cross-reactivity for PGE₂ and PGE₁ (Advanced Magnetics rabbit anti-PGE₂ antibody) was used at a final dilution of 1:21,000. Antibody-bound PG was separated from the free ligand with dextran-coated charcoal. The sensitivity of this assay was 7 pg/100 μ l (Hill & Larkins, 1989).

Adenylate cyclase assay

Intact-cell adenylate cyclase was assayed by measurement of cyclic [³H]AMP production after pre-labelling of cells with [2,8-³H]adenine (2 μ Ci/ml) for 2 h. After pre-labelling, the medium was replaced with medium containing 0.1% (w/v) BSA $\pm$ 3-isobutyl-1-methylxanthine (1 mM) for 20 min. When present, exogenous PGE₂ from a stock solution (10 pM–1 μ M), prepared in ethanol and stored under argon, was included for a further 30 min at 37 °C. Cyclic [³H]AMP formed over this period was measured after separation by sequential Dowex and alumina chromatography (Dunlop & Larkins, 1989).

Materials

Fura-2-AM and BAPTA-AM were obtained from Molecular Probes (Junction City, OR, U.S.A.); ET1 was obtained as a synthetic peptide (Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Trp) from Peptide Institute Inc. (Osaka, Japan); [2,8-³H]adenine, [5,6,8,11,12,14,15(n)-³H]PGE₂ and ⁴⁵Ca were from Amersham International (Amersham, Bucks., U.K.). All other chemicals were from Sigma (St. Louis, MO, U.S.A.).

RESULTS

Comparison of [Ca²⁺]_i and cell contractility in cells cultured in the presence and absence of added insulin

In mesangial-cell monolayers cultured in the presence of insulin, a dose-dependent rise in [Ca²⁺]_i was seen in response to AII (1 nM–1 μ M), PAF (0.1 nM–0.1 μ M) and ET1 (0.1 nM–0.1 μ M). In these monolayers loaded with fura-2 a rapid peak increase in cytosolic Ca²⁺ was seen after addition of these agonists, with maximal increases occurring from a basal value of 99 $\pm$ 4 nM to 196 $\pm$ 12, 240 $\pm$ 10 and 342 $\pm$ 12 nM in response to AII (0.1 μ M), PAF (0.1 μ M) and ET1 (10 nM) respectively, within 20–50 s of agonist addition, followed by a sustained elevation for up to 200 s (Fig. 1). This increase was seen in all mesangial-cell populations cultured in the presence of insulin, at either 0.7 nM or 7 μ M; thus all subsequent determinations were made with 0.7 nM-insulin. No difference in basal unstimulated [Ca²⁺]_i was seen between cells cultured with and without added insulin. The magnitude of the peak response was decreased significantly in those cells cultured without added insulin. The initial peak [Ca²⁺]_i reached is shown in Table 1. Insulin-deplete cells appear less sensitive to all agonists, requiring concentrations approximately one log order of magnitude greater than that required in cells cultured with insulin (0.7 nM) to cause a significant rise in [Ca²⁺]_i. A concomitant contractile response was seen in monolayer cells cultured with insulin, but not from insulin-deficient cultures, and when quantified in single cells

Fig. 1. Intracellular Ca^{2+} release in response to agonist addition to mesangial cells

Mesangial-cell monolayers were maintained in medium containing insulin (0.7 nM) on glass coverslips as described in the Materials and methods section. $[\text{Ca}^{2+}]_i$ was determined in fura-2-loaded monolayers in response to 0.1 μM -AII (a), 0.1 μM -PAF (b) and 10 nM-ET1 (c) present for 250 s of stimulation. The results shown are for a representative monolayer, with the calculated value for free $[\text{Ca}^{2+}]_i$ (nM) indicated for basal and peak values.

Table 1. $[\text{Ca}^{2+}]_i$ in fura-2-loaded monolayers of mesangial cells

Values are means $\pm$ S.E.M. for 10 (basal) or 6 (all others) estimations: ^a significant difference from insulin-replete (0.7 nM); $P < 0.05$; ^b first point with significant difference from basal value ($P < 0.05$).

Condition	Peak $[\text{Ca}^{2+}]_i$ (nM)	
	Insulin (0.7 nM)	Insulin-deficient
Basal	99 $\pm$ 4	94 $\pm$ 7
AII	(1 nM) 154 $\pm$ 11 ^b	114 $\pm$ 12 ^a
	(10 nM) 184 $\pm$ 17	124 $\pm$ 15 ^a
	(0.1 μM) 196 $\pm$ 12	142 $\pm$ 19 ^{a,b}
	(1 μM) 210 $\pm$ 11	164 $\pm$ 15 ^a
PAF	(0.1 nM) 162 $\pm$ 21 ^b	117 $\pm$ 12
	(1 nM) 194 $\pm$ 14	154 $\pm$ 14 ^b
	(10 nM) 232 $\pm$ 17	174 $\pm$ 13 ^a
	(0.1 μM) 240 $\pm$ 10	194 $\pm$ 19 ^a
ET1	(0.1 nM) 190 $\pm$ 14 ^b	171 $\pm$ 14
	(1 nM) 340 $\pm$ 17	290 $\pm$ 12 ^{a,b}
	(10 nM) 342 $\pm$ 12	299 $\pm$ 19
	(0.1 μM) 354 $\pm$ 16	224 $\pm$ 21 ^a

from these monolayers contraction was significantly greater for a given agonist concentration in insulin-replete cells. In all cases, a contractile response was reflected in a significant, maintained, decrease in cell length over the timed monitoring period (up to

Fig. 2. Contractility of individual mesangial cells cultured in the presence or absence of added insulin

The change in cell length was determined by video-microscopy of individual cells grown in monolayers in the presence of 0.7 nM-insulin (a, c, e) or insulin-free (b, d, f). At zero time monolayers were stimulated with 0.1 μM -AII (a, b), 0.1 μM -PAF (c, d) or 10 nM-ET1 (e, f). The results are means $\pm$ S.E.M. for 10 monolayer preparations at each condition.

300 s). The maximal decrease achieved with all agonists (17.6 $\pm$ 0.1 % of resting cell length) was significantly greater than the maximal response in insulin-deficient cells (10.4 $\pm$ 0.1 %; $P < 0.005$, $n = 50$) (Fig. 2).

Effect of modification of cellular $[\text{Ca}^{2+}]_i$ on contractile response of cells cultured in the presence of insulin

The mechanism(s) for $[\text{Ca}^{2+}]_i$ increase and effect on contraction were investigated further in insulin-replete monolayers. When the intracellular- Ca^{2+} buffer BAPTA was titrated into the cells as its acetomethoxy ester, an extracellular concentration of 10 μM prevented the agonist-induced $[\text{Ca}^{2+}]_i$ rise in response to AII and PAF (0.1 μM), but had no significant effect on the rise in $[\text{Ca}^{2+}]_i$ in ET1-stimulated monolayers. The peak $[\text{Ca}^{2+}]_i$ achieved was 92 $\pm$ 10, 101 $\pm$ 4 and 297 $\pm$ 17 nM respectively (Fig. 3). However, chelation of extracellular Ca^{2+} with EGTA inhibited significantly the rise in $[\text{Ca}^{2+}]_i$ in response to ET1, while suppressing only a late sustained phase of intracellular Ca^{2+} seen after AII or PAF (peak $[\text{Ca}^{2+}]_i$ was 142 $\pm$ 10, 194 $\pm$ 10, 224 $\pm$ 17 nM after ET1, AII or PAF respectively; Fig. 3). As shown in Table 2, maximal agonist-induced contraction, as change in cell length, was decreased after these manipulations to decrease either a peak or sustained $[\text{Ca}^{2+}]_i$ rise. The nifedipine analogue, calcium-channel agonist BAY K 8644, elicited only a small non-significant change in cell length (contraction) in these cells.

Comparison of cellular PGE levels and adenylate cyclase activity in cells cultured in the presence and absence of added insulin

Basal PGE levels, determined by radioimmunoassay, were significantly greater in cells cultured in the absence of insulin (41.1 $\pm$ 3.7 versus 26.0 $\pm$ 2.0 pg/30 min per μg of protein; $P < 0.01$; no added insulin versus insulin added in culture). There was a concomitant increase in cellular adenylate cyclase activity in cells cultured without added insulin (cyclic $[\text{H}^3]\text{AMP}$ formation 1810 $\pm$ 90 versus 1230 $\pm$ 74 d.p.m./100 μg of protein; $P < 0.005$; no added insulin versus insulin added in culture). A dose-dependent rise in whole-cell adenylate cyclase activity followed addition of exogenous PGE_2 (0.1 nM–1 μM) in both

Fig. 3. Effect of inclusion of Ca^{2+} -chelating agents BAPTA and EGTA on agonist-induced $[\text{Ca}^{2+}]_i$ in cells cultured with added insulin

Mesangial-cell monolayers were maintained with 0.7 nM-insulin alone (a, d, g) or pretreated with 10 μM -BAPTA-AM (b, e, h) or 4 mM-EGTA (c, f, i) for 20 min before stimulation with 0.1 μM -AII (a-c), 0.1 μM -PAF (d-f) or 10 nM-ET1 (g-i). The results shown are for representative monolayers with the calculated value for free $[\text{Ca}^{2+}]_i$ (nM) at the basal (unstimulated) and peak (agonist-induced) level indicated on the vertical axis.

Table 2. Maximal contraction in response to agonists in mesangial-cell monolayers cultured in the presence of insulin

Values are means $\pm$ S.E.M. for 10 (basal) or 6 (all others) estimations: ^a significant change in cell length compared with basal ($P < 0.05$, calculated from μm values); ^b and ^c significant difference from agonist alone ($P < 0.001$ and $P < 0.05$ respectively).

Condition	Cell length (% of resting)
Basal	100
AII (0.1 μM)	84.2 $\pm$ 1.7 ^a
+EGTA (4 μM)	91.4 $\pm$ 1.4 ^c
+BAPTA (10 μM)	89.5 $\pm$ 1.7
PAF (0.1 μM)	82.5 $\pm$ 2.1 ^a
+EGTA	92.1 $\pm$ 2.1 ^b
+BAPTA	89.4 $\pm$ 1.7 ^c
ET 1 (10 nM)	82.4 $\pm$ 1.9 ^a
+EGTA	91.4 $\pm$ 1.4 ^b
+BAPTA	83.4 $\pm$ 2.0
BAY K 8644 (10 μM)	94.1 $\pm$ 2.1

conditions of culture, with no difference in the sensitivity of adenylate cyclase activation in intact cells from either insulin-replete or -deficient culture conditions (K_a 7 nM or 8 nM respectively) (Fig. 4).

Effect of exogenous PGE_2 and 8BrCAMP on cell contractility in cells cultured with added insulin

To determine whether a raised basal level of PGE and adenylate cyclase activation could contribute to loss of cellular contractility in cells cultured without added insulin, modification of the contractile response in insulin-replete cells was investigated by pre-treatment with PGE_2 (0.1 nM–0.1 μM) or 8BrCAMP (1 nM–0.1 μM). In PGE_2 -pre-treated cells the contractile response was attenuated in response to all agonists (Fig. 5). A similar attenuation of the contraction of single cells stimulated with AII (0.1 μM) was seen after pre-treatment with 8BrCAMP (Fig. 5d). Under the experimental conditions (rigid support) it was not possible to determine if these pre-treatments had altered resting

Fig. 4. Effect of PGE_2 on adenylate cyclase activation in mesangial-cell monolayers

Mesangial-cell monolayers cultured in the presence of 0.7 nM-insulin (●) or without added insulin (○) were labelled with [2,8- ^3H]adenine and the production of cyclic [^3H]AMP was determined after stimulation with PGE_2 (0.1 nM–1 μM). Results are means $\pm$ S.E.M. for cyclic [^3H]AMP formation calculated as a percentage of maximal formation [21476 $\pm$ 210 d.p.m./100 μg of protein (insulin added) and 22424 $\pm$ 242 d.p.m./100 μg of protein (no added insulin)] at 1 μM - PGE_2 . A value for K_a of 7 nM (insulin added) and 8 nM (no insulin added) was calculated from the value of 50% of maximal stimulation in 10 preparations.

cell length. At maximal agonist concentration these pre-treatments did not cause a significant decrease in the peak $[\text{Ca}^{2+}]_i$ response (Table 3). A significant rise in $[\text{Ca}^{2+}]_i$ was seen at higher concentrations of PGE_2 ($> 1 \mu\text{M}$; results not shown), but the contractile response remained depressed.

Effect of indomethacin on PGE levels and AII-induced cell contractility in cells cultured in the presence and absence of added insulin

To determine the effect of decreased endogenous PGE on cell

Fig. 5. Effect of preincubation with PGE_2 and 8BrcAMP on agonist-induced contraction of individual cells from mesangial-cell monolayers cultured in the presence of insulin

Mesangial-cell monolayers were preincubated with PGE_2 (0.1 nM – $0.1 \mu\text{M}$), and cell length of individual cells was determined after stimulation with $0.1 \mu\text{M}$ -AII (a), $0.1 \mu\text{M}$ -PAF (b) or 10 nM -ET1 (c). In (d) results are shown for monolayers preincubated with 8BrcAMP (1 nM – $1 \mu\text{M}$) and subsequent stimulation with AII ($0.1 \mu\text{M}$). The results are means $\pm$ S.E.M. for the calculated percentage decrease in cell length at 250 s after agonist alone ($\square$) or agonist addition to PGE_2 - or 8BrcAMP-pre-treated cells ($\blacksquare$), for 10 determinations from multiple monolayers.

Table 3. Peak $[\text{Ca}^{2+}]_i$ in response to agonists in mesangial-cell monolayers cultured in the presence of insulin

Values are means $\pm$ S.E.M. for 10 (basal) or 6 (all others) estimations:
^asignificant difference from basal ($P < 0.05$).

Condition	Peak $[\text{Ca}^{2+}]_i$ (nM)
Basal	99 ± 4
AII ($0.1 \mu\text{M}$)	196 ± 12^a
+ PGE_2 (10 nM)	201 ± 14
+ 8BrcAMP ($1 \mu\text{M}$)	190 ± 21
PAF ($0.1 \mu\text{M}$)	20 ± 10^a
+ PGE_2	214 ± 10
+ 8BrcAMP	196 ± 7
ET1 (10 nM)	342 ± 12^a
+ PGE_2	340 ± 21
+ 8BrcAMP	336 ± 4

Table 4. Effect of indomethacin on AII-induced cell contraction

Values are means $\pm$ S.E.M. for six estimations. The maximal decrease in cell length was determined 200–250 s after application of $0.1 \mu\text{M}$ -AII and expressed as the percentage decrease in resting cell length:
^asignificant difference from pre-treatment with 0.7 nM -insulin ($P < 0.01$); ^bsignificant difference from no added insulin alone ($P < 0.005$).

Pre-treatment	Maximal decrease in cell length after $0.1 \mu\text{M}$ -AII (%)
Insulin (0.7 nM)	15.8 ± 1.4
+ indomethacin ($1 \mu\text{M}$)	17.4 ± 2.1
No added insulin	10.2 ± 1.2^a
+ indomethacin ($1 \mu\text{M}$)	17.8 ± 1.4^b

contractility, cells were pre-incubated with indomethacin ($1 \mu\text{M}$) for 18 h. This pre-treatment significantly decreased basal PGE production in cells cultured in the presence (26.0 ± 2.0 to $2.9 \pm 1.0 \text{ pg/30 min per } \mu\text{g}$ of protein; $P < 0.001$) or in the absence of added insulin (41.1 ± 3.7 to $4.7 \pm 0.7 \text{ pg/30 min per } \mu\text{g}$; $P < 0.001$). As indicated above, it was not possible to determine if indomethacin pre-treatment had altered resting (basal) cell length. However, measurement of cell length after acute addition of AII ($0.1 \mu\text{M}$) showed that indomethacin pre-treatment did not alter the contractile potential of insulin-replete cells, but restored contractility to cells cultured in the absence of insulin, so that there was no significant difference in the maximal decrease in cell length between insulin-replete and -deficient cells after indomethacin pre-treatment (Table 4).

Comparison of $\text{Ins}(1,4,5)\text{P}_3$ -induced $^{45}\text{Ca}^{2+}$ release from permeabilized cells

To determine whether raised basal levels of PGE and thus adenylate cyclase activation with subsequent increase in cyclic AMP could contribute to the altered sensitivity of agonist-induced Ca^{2+} release seen in insulin-deficient cells, $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release was investigated in permeabilized cells. In saponin-permeabilized cells which had been pre-treated with PGE_2 (10 nM) or 8BrcAMP (1 nM), concentrations which produced an equivalent attenuation of cell contraction in insulin-replete cells (Fig. 5), ATP-dependent $^{45}\text{Ca}^{2+}$ uptake was significantly increased as compared with the uptake over 6 min in control cells cultured with added insulin (Table 5). The uptake in these insulin-replete cells is shown in Fig. 6 together with the release of $^{45}\text{Ca}^{2+}$ in response to $\text{Ins}(1,4,5)\text{P}_3$ ($1 \mu\text{M}$). This release was prevented by the concurrent presence of heparin ($10 \mu\text{g/ml}$). Over a 30 s stimulation period $^{45}\text{Ca}^{2+}$ was released from all permeabilized cells in response to exogenous $\text{Ins}(1,4,5)\text{P}_3$ in a dose-dependent manner, with a difference in the sensitivity of the release process demonstrated by the calculated EC_{50} values (concn. giving half-maximal effect) for each pretreatment, which were significantly different as compared with the untreated insulin-replete cells (Table 5). A similar difference in sensitivity was seen in permeabilized cells taken from insulin-deficient monolayer cultures, although, unlike PGE_2 and 8BrcAMP pretreatments, no significant difference in $^{45}\text{Ca}^{2+}$ uptake could be demonstrated (Table 5). However, a significant decrease in $\text{Ins}(1,4,5)\text{P}_3$ -sensitivity was apparent in insulin-deficient cells, and the calculated EC_{50} value for these cells (Table 5) is quantitatively similar to that seen after PGE_2 pre-treatment in cells cultured with added insulin. In all cases the $^{45}\text{Ca}^{2+}$ release in

Table 5. Effect of PGE₂ and 8BrcAMP on Ca²⁺ uptake and Ins(1,4,5)P₃-induced Ca²⁺ release in permeabilized cells

Values are expressed as means $\pm$ S.E.M. for 10 experimental determinations: ^asignificant difference from untreated ($P < 0.05$), ^b $P < 0.005$; ^csignificant difference from insulin-replete ($P < 0.01$).

Pretreatment	Other addition	ATP-dependent ⁴⁵ Ca ²⁺ uptake (nmol/100 μ g of protein)	Ins(1,4,5)P ₃ -induced ⁴⁵ Ca ²⁺ release [10 ⁻⁷ $\times$ EC ₅₀ (M)]
Insulin (0.7 nM)	Nil	0.53 $\pm$ 0.03	0.91 $\pm$ 0.21 ^a
	PGE ₂ (10 nM)	0.74 $\pm$ 0.07 ^a	1.82 $\pm$ 0.24
	8BrcAMP (1 nM)	0.72 $\pm$ 0.06 ^b	4.57 $\pm$ 0.31 ^b
No added insulin	Nil	0.56 $\pm$ 0.07	2.00 $\pm$ 0.61 ^c

Fig. 6. Time course of Ca²⁺ uptake and release in permeabilized insulin-replete mesangial cells cultured in the presence of insulin

Ca²⁺ uptake was measured in permeabilized cells in the presence of ATP. At 360s 1 μ M-Ins(1,4,5)P₃ was added alone (●) or in the presence of heparin (10 μ g/ml) (○), and cellular ⁴⁵Ca²⁺ content was determined at 10 s intervals. The results are the means of quadruplicate determinations.

response to Ins(1,4,5)P₃ could be prevented by the concurrent presence of heparin (results not shown).

DISCUSSION

The results of the present study confirm the considerable experimental evidence describing the effects of AII (Pfeilschifter & Bauer, 1986; Takeda *et al.*, 1988), PAF (Kester *et al.*, 1987; Boneventre *et al.*, 1988) and ET1 (Badr *et al.*, 1989; Simonson *et al.*, 1989) to raise [Ca²⁺]_i within mesangial cells, indicating that this is promoted by a receptor-coupled mechanism with initial release of Ca²⁺ from intracellular stores. For these agonists, hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C, demonstrated to be pertussis-toxin-sensitive after AII (Pfeilschifter & Bauer, 1986; Shin *et al.*, 1987) and ET1, provides Ins(1,4,5)P₃ for release of Ca²⁺ from intracellular sites (Shin *et al.*, 1987; Badr *et al.*, 1989; Simonson *et al.*, 1989). A voltage-dependent mechanism for Ca²⁺ entry has been proposed in coronary-artery smooth-muscle cells in response to ET1, based on nifedipine sensitivity (Yanagisawa *et al.*, 1988). However, although the contractile response to ET1 appears mainly dependent on entry of extracellular Ca²⁺, this cannot be attributed to a voltage-sensitive Ca²⁺ mechanism in mesangial cells, as demonstrated by a lack of BAY K 8644-sensitive Ca²⁺ entry. This is in agreement with the proposal for other smooth-muscle types that Ca²⁺ entry is through an alternative receptor-operated Ca²⁺ influx (Hirata *et al.*, 1988; Secrest & Cohen, 1989; Mitsuhashi *et al.*, 1989).

The present study confirms further that considerable attenuation of contractile responsiveness can result from exposure of mesangial-cell populations to exogenous prostanoids or by the addition of cyclic AMP to mimic the effect of PGE₂ activation of adenylate cyclase. Contractility measurements in many of these previous studies have drawn attention to the heterogeneity of the contractile response (Schlondorff *et al.*, 1984; Ardaillou *et al.*, 1985; Foidart & Mahieu, 1986; Scharschmidt *et al.*, 1986; Takeda *et al.*, 1988) and have shown that the addition of prostanoids results in a lower proportion of contractile cells, and, conversely, inhibition of metabolism of endogenous arachidonic acid to prostanoids with indomethacin allows a greater number of cells within a population to contract. The present study examines a mechanism by which individual cellular contractility may be altered. Investigation of individual cell contractility indicates that the sensitivity of a potentially contractile cell is decreased in insulin-deficient culture conditions and is restored by indomethacin pre-treatment. This study also shows that, although a rise in [Ca²⁺]_i, whether elicited by Ca²⁺ from intracellular sources (AII or PAF) or dependent mainly on extracellular Ca²⁺ (ET1) is sufficient to induce a contractile response in insulin-replete cultures, this does not suffice under insulin-deficient conditions. Treatment with exogenous PGE₂ gave a decrease of similar magnitude to that with culture in insulin-deficient conditions in all agonist-stimulated cells. Pretreatment with 8BrcAMP was the most effective means to decrease cell contractile potential. It is not known if the small residual contraction achieved (less than 5% of cell length) may be attributed to a contractile event or may be the result of a cyclic AMP-induced shape change due to focal proteolysis at cellular adhesion plaques, shown previously for mesangial cells under similar conditions of culture (Kriesberg & Venkatachalam, 1986).

The demonstration that cells cultured in the absence of insulin show an increase in endogenous prostanoid production prompted an investigation of the effects of a prostanoid (PGE₂) and a cyclic nucleotide (8BrcAMP) on intracellular Ca²⁺ availability. In permeabilized cells, PGE₂ and 8BrcAMP altered intracellular organelle ⁴⁵Ca²⁺ uptake and subsequent Ins(1,4,5)P₃-sensitive Ca²⁺ release. These findings are very similar to the findings in neural tissue, where both increased uptake and decreased release of ⁴⁵Ca²⁺ followed cyclic nucleotide exposure (Supattapone *et al.*, 1988) and were shown further to be associated with cyclic-AMP-dependent protein kinase phosphorylation of the cerebellar Ins(1,4,5)P₃-binding protein or receptor (Ferris *et al.*, 1989). Activation of a similar mechanism by raised cyclic AMP levels (PGE₂-elicited or after 8BrcAMP) in mesangial cells may explain the lack of sensitivity of the contractile response to AII and PAF, but not the lack of contractility seen in the presence of cyclic AMP generation in ET1-stimulated cells, where a major increase in [Ca²⁺]_i appears to be from extracellular sources. That ET1-stimulated contractility is decreased may be an indication of

a second cyclic-AMP-dependent mechanism contributing to decreased contractile responsiveness in these cells. Increased cyclic AMP levels have been proposed to decrease the sensitivity of myosin light-chain kinase to Ca^{2+} activation in smooth-muscle cells (Adelstein *et al.*, 1978). Alternatively, $\text{Ins}(1,4,5)\text{P}_3$ -promoted Ca^{2+} release may continue concomitant with Ca^{2+} entry after ET1 treatment (Badr *et al.*, 1989). The relative lack of responsiveness to exogenous $\text{Ins}(1,4,5)\text{P}_3$ is unlikely to be due to increased metabolism of $\text{Ins}(1,4,5)\text{P}_3$, as exposure to cyclic AMP (as dibutyryl cyclic AMP) does not alter the activity of an hepatic cytosolic $\text{Ins}(1,4,5)\text{P}_3$ kinase (Biden *et al.*, 1988). In cells cultured without added insulin (or in the presence of cyclic AMP) the relative lack of response of intracellular Ca^{2+} to $\text{Ins}(1,4,5)\text{P}_3$ may be most marked at sub-maximal agonist concentrations.

Insulin receptors have been shown in isolated rat glomeruli (Kurokawa *et al.*, 1980) and in cultured mesangial cells (Arnqvist *et al.*, 1988). In streptozotocin-diabetic rat glomeruli (insulinopaenic conditions), raised prostaglandin levels are decreased by the addition of insulin (Kriesberg & Patel, 1983; Craven *et al.*, 1987). Raised phospholipase A_2 activity has been demonstrated in glomeruli from such insulinopaenic diabetic rats (Craven *et al.*, 1988). The mechanism(s) by which phospholipase A_2 is activated in diabetic glomeruli remains to be elucidated, although increased activity of protein kinase C may contribute in hyperglycaemic conditions (Craven & De Rubertis, 1989) with stimulatory diacylglycerol derived from phosphatidic acid synthesized *de novo* (Craven *et al.*, 1990). This stimulation may be by direct action on the enzyme (Halenda *et al.*, 1989) or by the removal of the inhibitory action of lipocortin(s) through protein-kinase-C-mediated phosphorylations. Two Ca^{2+} -dependent phospholipid-binding lipocortins, p35 and p36, are substrates for protein kinase C phosphorylation (Isacke *et al.*, 1986; Khanna *et al.*, 1986). The present study indicates that high glucose levels in culture may not be sufficient, as mesangial cells cultured in both the presence and the absence of added insulin were maintained at an elevated glucose concentration. By analogy with other cell systems, it is possible that insulin may redirect glucose metabolism and/or act to lower the production of diacylglycerol by decreasing phosphatidate phosphohydrolase activity (EC 3.1.3.4), the final enzyme step in glucose-derived diacylglycerol production. In hepatic microsomes this enzyme is activated by the presence of cyclic AMP (Pittner *et al.*, 1985) or in the diabetic condition (Murthy & Shipp, 1979) and is inhibited by insulin (Pittner *et al.*, 1985). Insulin may also act to decrease cyclic AMP by stimulation of cyclic AMP phosphodiesterase, as shown in hepatic plasma membranes (Houslay, 1986), and thus may attenuate any subsequent cyclic AMP actions, including the recently described effect of cyclic AMP to stimulate group II phospholipase A_2 mRNA synthesis, shown in vascular smooth-muscle cells (Nakano *et al.*, 1990). The findings of decreased PGE levels in insulin-replete cultures indicate that insulin may be responsible for the decrease in phospholipase A_2 and subsequent prostanoid production and the maintenance of contractility in mesangial cells in the absence of vasoactive agents.

As raised PGE_2 production may follow Ca^{2+} -dependent activation of phospholipase A_2 in PAF-, platelet-derived-growth-factor- (Boneventre *et al.*, 1988) and endotoxin-lipopolysaccharide-stimulated (Wang *et al.*, 1988) mesangial cells in culture, it suggests this as a mechanism for potential negative regulation of contractility in response to varied mesangial- and intra-glomerular-derived vasoactive compounds. The present studies in cultured mesangial cells indicate that insulinopaenic conditions will accentuate this mechanism. The findings offer a means by which elevated glomerular prostaglandin production and insulin deficiency may alter mesangial-cell reactivity, with consequent effects on glomerular filtration and permeability.

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