Epidermal growth factor is synergistic with phorbol esters and vasopressin in stimulating arachidonate release and prostaglandin production in renal glomerular mesangial cells

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Epidermal growth factor (EGF) is produced in large quantities by the kidney. We identified EGF-binding sites on cultured rat renal glomerular mesangial cells. These cells serve as a model system for the investigation of renal prostaglandin biosynthesis. Since EGF has been shown to modulate phospholipase activity in other cell lines, we studied the ability of EGF to increase arachidonate release and prostaglandin E_2 (PGE_2) production in mesangial cells. We found that EGF stimulated arachidonate release and PGE_2 production in the presence of the Ca^{2+} ionophore A23187. This stimulation was markedly potentiated by the addition of phorbol myristate acetate (PMA), which activates protein kinase C. However, down-regulation of protein kinase C by prolonged PMA treatment did not block the ability of EGF to stimulate PGE_2 production in the presence of A23187. EGF also markedly potentiated the stimulation of PGE_2 production by vasopressin, which increases intracellular Ca^{2+} and activates protein kinase C in these cells. The stimulatory effects of EGF were not the result of prolongation or enhancement of an increase in intracellular Ca^{2+} produced by ionophore or vasopressin. Furthermore, the synergistic interaction of EGF with PMA and vasopressin occurred despite the fact that these agents markedly decreased EGF binding in mesangial cells, presumably owing to protein-kinase-C-mediated phosphorylation of the EGF receptor. We conclude that there exists a distinct pathway for EGF-stimulated arachidonate release and PGE_2 production in rat renal glomerular mesangial cells, which is synergistic with, but not dependent on, activation of protein kinase C. In contrast with long-term mitogenic responses to EGF, this rapid response may allow delineation of the membrane phospholipid changes and signalling steps involved in this aspect of EGF action.

INTRODUCTION

Epidermal growth factor (EGF) is a 53-amino-acid peptide with potent mitogenic properties. Cellular responses to EGF can be divided into early responses such as enhanced ion transport and late responses such as enzyme induction [1]. One of the early effects seen within seconds of EGF binding to its receptor is protein phosphorylation at tyrosine residues [2]. Great experimental effort has been expended to identify the protein substrates of the EGF-receptor tyrosine kinase and determine their physiological role. Pepinsky and co-workers have sequenced a 35 kDa protein [3], which Cohen and co-workers had previously identified as a major substrate of EGF tyrosine kinase [4]. This protein was identified as lipocortin, a family of proteins thought to have a role in the modulation of phospholipase A_2 activity and therefore arachidonic acid release and prostaglandin production. It has been postulated that phosphorylation of lipocortin alters its phospholipase inhibitory action; however, a direct link has been difficult to demonstrate [5].

Several reports have suggested that EGF alters prostaglandin production by cells in culture [6–10]. The effects of EGF on prostaglandin production may have particular physiological relevance in the kidney, because

many aspects of renal function are controlled by prostaglandins [11], and EGF is produced in large quantities by the kidney [12,13]. Therefore we used cultured rat renal glomerular mesangial cells to study the effects of EGF on arachidonate release and prostaglandin production. These cells have been well characterized and used extensively as a model system for investigating the regulation of renal prostaglandin biosynthesis by vasoactive hormones such as angiotensin II and vasopressin [14]. The findings in the present study suggest a distinct pathway for stimulation of arachidonate release and PGE_2 synthesis in these cells which is synergistic with, but not dependent on, activation of protein kinase C. This effect of EGF also served to augment the stimulation of PGE_2 production by vasopressin.

EXPERIMENTAL

Materials

EGF was purified from mouse submaxillary glands as previously described [15] and utilized as a stock solution of 0.1 μ M in 50 mM-acetic acid. Obtained from GIBCO (Burlington, Ont., Canada) were cell-culture media, sera and enzymes. PGE_2 radioimmunoassay kits were obtained from New England Nuclear (Bedford, MA,

Abbreviations used: EGF, epidermal growth factor; PMA, phorbol myristate acetate; PGE_2 , prostaglandin E_2 .

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U.S.A.). All other reagents were from Sigma (St. Louis, MO, U.S.A.). Stock solutions of A23187 (1 mM) and PMA (1 mg/ml) were prepared in dimethyl sulphoxide, which in the concentrations used had no effect on PGE₂ production.

Cell culture

Homogeneous cultures of renal glomerular mesangial cells were derived from collagenase-treated glomeruli isolated from young Sprague-Dawley rats, and were characterized as previously described [16]. Mesangial cells were spindle-shaped in appearance, contracted in response to vasopressin and angiotensin II, and demonstrated positive immunocytochemical staining for desmin and vimentin and stained negative for cytokeratin. Cloned populations derived from single colonies were used throughout. Cell cultures were maintained in Corning 75 cm² flasks in DME (Dulbecco's modified Eagle medium) supplemented with 20% (v/v) fetal bovine serum and incubated at 37 °C with 5% CO₂.

¹²⁵I-EGF binding

Radioligand binding was performed in adherent cells as previously described [17]. Cells were subcultured and grown to confluence in 16 mm-diam. wells. The medium was aspirated and the cells were washed twice in incubation buffer (DME containing 30 mM-Hepes, pH 7.5, and 1 mg of bovine serum albumin/ml) and then incubated in the same buffer containing different concentrations of added ¹²⁵I-EGF (sp. radioactivity 60 µCi/µg) at 37 °C for 1 h. At the end of incubation, cells were rapidly washed six times with ice-cold incubation buffer, the cells were dissolved in 0.5 M-NaOH, and cell-associated radioactivity was determined in a γ-radiation counter. Non-specific binding was determined in the presence of excess (0.5 µM) unlabelled EGF at each concentration of ¹²⁵I-EGF and subtracted from total radioactivity to yield specific binding. Non-specific binding was always less than 5% of total radioactivity.

PGE₂ production

For these experiments, cells were plated at a density of 4×10^5 cells per 100 mm dish in 20% fetal bovine serum and 2 mM-butyrate [18] and studied at 48 h. Cells were washed twice and equilibrated in incubation buffer for 1 h at 37 °C. The incubation buffer was aspirated and replaced with fresh incubation buffer containing 2 mM-GSH and the agents to be tested. At the end of 30 min, the medium was removed and samples were assayed in duplicate for PGE₂ by radioimmunoassay. Cells were washed with phosphate-buffered saline (Dulbecco's) and cell protein was assayed by the Lowry method [19]. Qualitatively similar results were obtained in cells grown without butyrate.

Arachidonate release

Cells were grown to confluence in 100 mm dishes for 6 days in medium without butyrate but supplemented with 0.5 µCi of [³H]arachidonate. Cells were removed from serum for 24 h in the continued presence of labelled arachidonate. Cells were then washed and equilibrated for 2 h at 37 °C in incubation buffer. The buffer was aspirated and replaced with 5 ml of incubation buffer containing the agents to be tested. At the end of a 30 min incubation at 37 °C, the buffer was removed, and added to a test tube containing 11 µl of 88% (w/v) formic acid.

Then 2 ml of ice-cold methanol was added to the cells, and the dish was scraped and the suspension added to the acidified buffer. Next 3 ml of chloroform/methanol (1:1.2, v/v) and then an additional 3 ml of chloroform was added to the cellular suspension. After mixing and centrifugation the organic phase was removed, divided into two aliquots and dried under nitrogen. The following standards were prepared for t.l.c.: triolein in propan-2-ol, 300 mg/dl; diolein in chloroform, 100 mg/2 ml; arachidonate in ethanol, 10 mg/2 ml; and mono-oleoyl-*rac*-glycerol in ethanol, 20 mg/2 ml. The samples were dissolved in 40 µl of triolein, 5 µl of diolein, 10 µl of arachidonate and 10 µl of mono-oleoyl-*rac*-glycerol standard solutions. Samples and standards were applied to pre-activated Whatman LK5 DF t.l.c. plated and run for approx. 90 min. The solvent consisted of 100 ml of the organic phase from a mixture of 55 ml of ethyl acetate, 75 ml of iso-octane, 8 ml of acetic acid and 100 ml of water. The plates were dried and developed in iodine. The free fatty acid band was scraped from each lane, and then the remainder of the lane was scraped off and counted for radioactivity in a liquid-scintillation counter. The results were expressed as percentage of total lane radioactivity in the free fatty acid band.

Fluorimetric measurements of cytosolic Ca²⁺

These measurements were performed as previously described [20], except that Indo-I-AM was used in place of quin2 as the Ca²⁺-sensitive fluorescent probe and cells were studied while adherent to glass coverslips. Briefly, cells were grown to confluence on rectangular coverslips and incubated with Indo-I-AM (2.5 µM) at 37 °C for 40 min. Loaded cells were washed free of extracellular dye, and coverslips were placed in Hepes-buffered physiological salt solution (137 mM-NaCl, 2.7 mM-KCl, 11.1 mM-glucose, 10 mM-Hepes, 0.5 mM-CaCl₂, 1.0 mM-MgCl₂, pH 7.4) for measurement of fluorescence in a Hitachi F-4000 fluorescence spectrophotometer equipped with a thermostatically controlled cuvette, continuous stirring device and auto-injection system. Excitation was at 355 nm and emission recorded at 400 nm. Calibration of fluorescence intensity to Ca²⁺ measurements was as previously described, auto-fluorescence being subtracted from all fluorescence values before calculation [21]. The non-fluorescent Ca²⁺ ionophore ionomycin was used in place of A23187 for these experiments.

Protein kinase C down-regulation

For experiments on this, mesangial cells were plated at 200 000 cells per 60 mm dish. After 24 h the standard medium with 20% serum and 2 mM-butyrate was removed, and DME with 5% serum, 2 mM-butyrate, penicillin/streptomycin, and dimethyl sulphoxide (final concn. 0.03%) or PMA (300 ng/ml) added to the cells. After a further 24 h in PMA or dimethyl sulphoxide, cells were washed four times with incubation buffer, and prostaglandin production was assessed as above.

RESULTS

The EGF concentration-dependence for cell-associated ligand accumulation (binding) is shown in Fig. 1. In the absence of added PMA, half-maximal binding occurred at approx. 1 nM-EGF and maximal binding at 10 nM-EGF. Addition of PMA markedly decreased EGF binding at all concentrations of EGF tested, such that

Fig. 1. ^{125}I -EGF binding to rat mesangial cells in culture

Cells were incubated for 1 h with ^{125}I -EGF in the presence (○) or absence (●) of PMA (100 ng/ml), and binding was measured as described in the Experimental section. Each point represents the mean of three wells, and these curves are representative of the results from two experiments.

saturable binding could not be achieved with the highest concentrations of ligand used (25 nM). Because internalization occurs at 37 °C, binding represents both surface-bound and internalized ligand, and therefore true surface-receptor-binding parameters cannot be rigorously derived. With this reservation, Scatchard analysis of the data shown in Fig. 1 revealed approx. 96 000 receptors per cell with a K_d of 2.2 nM, which changed to 59 000 and 8.0 nM respectively with PMA treatment. This effect of PMA to decrease EGF binding was observed with as little as 1.0 ng of PMA/ml and is consistent with the known effect of protein kinase C to phosphorylate the EGF receptor and to decrease markedly its binding affinity [22]. Vasopressin also markedly decreased EGF binding in a manner similar to the effect of PMA (results not shown).

The effects of EGF (10 nM) on PGE_2 production are shown in Fig. 2, and the EGF concentration-dependence is shown in Fig. 3. In the absence of A23187, neither EGF nor PMA resulted in significant stimulation of PGE_2 production. In the presence of A23187, both EGF and PMA resulted in marked stimulation of PGE_2 production, and in combination EGF and PMA were synergistic. The combination of EGF and PMA with A23187 resulted in a greater than 7-fold stimulation of PGE_2 production over that with A23187 alone, and greater than 15-fold stimulation over control. The stimulatory effect on PGE_2 production in the presence of A23187 was evident at the lowest concentration of EGF tested (0.1 nM), and a maximal effect was seen at approx. 1 nM (Fig. 3). The addition of PMA markedly potentiated the stimulatory effect of EGF at every concentration of EGF tested, although PMA decreased EGF binding, as shown in Fig. 1.

Vasopressin stimulates hydrolysis of membrane phosphoinositides in mesangial cells in a V_1 -receptor-specific manner [23]. As a result inositol trisphosphate is released, resulting in a rise in cytosolic free Ca^{2+} [24], and

Fig. 2. PGE_2 production by rat mesangial cells in culture

Cells were incubated for 30 min either in the absence (□) or the presence (▨) of EGF (10 nM), with the other additives indicated (control, C; PMA, 100 ng/ml; A23187, 100 nM), and PGE_2 production was measured as described in the Experimental section. Results are means $\pm$ S.E.M. for six experiments; * $P < 0.01$ versus control, ** $P < 0.01$ versus all other groups.

Fig. 3. EGF concentration-dependence for stimulation of PGE_2

Cells were incubated without (○) or with (●) PMA (100 ng/ml) in the presence of A23187 (100 nM) for 30 min, and PGE_2 production was measured as described in the Experimental section. Curves are from one experiment, where each point represents the mean of two dishes. These curves are representative of the data from three separate experiments. The half-maximally stimulating concentrations of EGF from the three experiments were 0.52 ± 0.13 nM in the absence of added PMA and 0.58 ± 0.18 nM with PMA (means $\pm$ S.E.M.).

Fig. 4. Synergistic effects of EGF and vasopressin on PGE₂ production

Cells were incubated for 30 min with the concentrations of vasopressin shown, either with (●) or without (○) 10 nM-EGF, and PGE₂ production was measured. Results represent means $\pm$ S.E.M. for six experiments; * P < 0.01 versus the corresponding vasopressin concentration without EGF.

diacylglycerol is produced, which is known to activate protein kinase C [25]. It was therefore decided to determine whether this synergistic effect of EGF with PMA in the presence of A23187 would also be evident with vasopressin. As shown in Fig. 4, vasopressin alone stimulated PGE₂ production in a concentration-dependent manner. At each of three concentrations of vasopressin tested, EGF potentiated the stimulatory effect of vasopressin.

Studies were done to determine if the effects of EGF were exerted at the level of arachidonate release or at the level of conversion of arachidonate into PGE₂. Arachidonate (1 μ g/ml) increased PGE₂ production from 8.8 ± 1.2 to 75 ± 2.6 ng/30 min per mg of protein, but there was no further enhancement by 10 nM-EGF (77 ± 3.4 ng/30 min per mg of protein; mean $\pm$ S.E.M., $n = 8$). This indicates that the effect of EGF to stimulate PGE₂ production does not occur primarily at the level of cyclo-oxygenase. In contrast, 10 nM-EGF did cause a significant enhancement of arachidonic acid release in cells prelabelled with [³H]arachidonate (Table 1).

Studies were also done in order to discern that the effect of EGF on arachidonate release and PGE₂ production was not the result of enhancement of the rise in cytosolic Ca²⁺ that occurs with Ca²⁺ ionophore or vasopressin. We have previously characterized the cytosolic Ca²⁺ response in these cells [20]. When the intracellular fluorescent Ca²⁺ probe Indo-1 was used as described in the Experimental section, EGF (80 nM) did not raise baseline cytosolic Ca²⁺, nor enhance the intracellular Ca²⁺ increase induced by ionomycin or vasopressin.

Table 1. Effect of EGF on arachidonic acid release in mesangial cells

Mesangial cells were loaded with [³H]arachidonic acid, and release as free fatty acid was measured as outlined in the Experimental section. Concentrations of ligands were as follows: 1 μ M-A23187, 10 nM-EGF, 1 μ M-vasopressin. Results are expressed as means $\pm$ S.E.M.; * P < 0.05 compared with corresponding ligand without EGF.

	Release of ³ H as free arachidonic acid (%)	<i>n</i>
Control	2.73 $\pm$ 0.24	6
A23187	4.39 $\pm$ 0.51	5
A23187 + EGF	6.35 $\pm$ 0.44*	5
Vasopressin	10.44 $\pm$ 0.51	5
Vasopressin + EGF	18.43 $\pm$ 0.69*	5

Fig. 5. Effects of protein kinase C down-regulation on PGE₂ production

In (a) cells were treated for 24 h with dimethyl sulphoxide (0.03%), and in (b) cells were treated for 24 h with PMA (300 ng/ml). After 24 h PGE₂ production was assayed in response to 100 nM-A23187 (A), A23187 + PMA (100 ng/ml) (A+P), A23187 + 10 nM-EGF (A+E), or A23187 + PMA + EGF (A+P+E). Results are means $\pm$ S.E.M. for six experiments; * P < 0.01 versus A23187 alone.

Finally, experiments were undertaken to examine the EGF response in cells which had undergone prolonged exposure (24 h) to PMA in order to down-regulate protein kinase C. Down-regulation of protein kinase C was evident by the loss of the ability of PMA added acutely to decrease EGF binding [26]. In non-down-regulated cells, PMA decreased the binding of EGF (1 nM) by $78.8 \pm 0.8\%$ ($n = 3$), whereas in down-regulated cells there was an insignificant increase in binding of $2.4 \pm 0.6\%$ ($n = 3$). This is consistent with loss of stimutable protein kinase C in the down-regulated cells. Despite the apparent loss of protein kinase C in the

down-regulated cells, EGF still resulted in stimulation of PGE_2 production in the presence of A23187 (Fig. 5). However, as expected in the down-regulated cells, PMA was completely ineffective, neither augmenting A23187-induced PGE_2 production nor potentiating the effect of EGF.

DISCUSSION

Mesangial cells subserve a number of functions in the glomerulus, including regulation of glomerular filtration [27], prostaglandin release [11], immune function [28] and elaboration of glomerular matrix [29]. Cultured rat glomerular mesangial cells have been used as a model system for studying the regulation of renal prostaglandin production by vasoactive hormones and other agents [14]. The current study demonstrates a stimulatory effect of EGF on arachidonate release and PGE_2 production in these cells. These effects were seen under conditions associated with a rise in intracellular Ca^{2+} , but EGF did not act by further elevating intracellular Ca^{2+} . Although this effect of EGF was not dependent on protein kinase C, as demonstrated in the PMA down-regulation experiments, EGF stimulation was further potentiated by activation of protein kinase C.

The mutual interaction of EGF-mediated effects with activation of protein kinase C has been studied in a number of cell types [30]. The best characterized interaction is protein-kinase-C-mediated phosphorylation of the threonine-654 residue on the EGF receptor, which decreases ligand-binding affinity [22]. This probably explains the decrease in EGF binding to mesangial cells which occurred in PMA-treated cells. Despite a lowering of EGF-binding affinity, protein kinase C activation has been shown to be synergistic with EGF in mitogenesis in some [31], but not all [32], cell types. The synergism between PMA and EGF in mitogenesis occurs over many hours, and has been attributed to increased cellular uptake of EGF [33] and increased EGF-receptor synthesis [34]. Although this may explain the synergistic effect of PMA on late responses to EGF, the current study demonstrates a rapid physiological response to EGF and a rapid synergistic effect with PMA. A similar effect of PMA to augment α -transforming-growth-factor-stimulated arachidonate release from cultured hepatic cells was reported by Levine and co-workers [35]. The ability of PMA to augment the stimulatory effect of EGF, despite a decrease in EGF binding, may be explained by a different time course for the two effects. Since the threonine-654 phosphorylation of the EGF receptor and the decrease in binding occur within minutes, the synergism with PMA must occur rapidly, consistent with the results in the current study. Less likely, the coupling relationship between EGF-receptor occupancy and receptor-mediated signalling might be augmented and become markedly non-linear in the presence of PMA. The current study does not distinguish between these possibilities.

It should be noted that prolonged exposure to EGF has been shown to stimulate cyclo-oxygenase activity [10]. However, in the current study the effects of EGF took place over 30 min, and no effect of EGF on conversion of arachidonate into PGE_2 was observed. Rather, the stimulatory effect of EGF on PGE_2 production occurred at the level of arachidonate release. Both phospholipase C- and phospholipase A_2 -mediated

pathways for arachidonate release have been identified in glomerular mesangial cells [36]. Vasoactive hormones such as vasopressin activate phospholipase C, which acts on membrane polyphosphoinositides to produce inositol trisphosphate and diacylglycerol [24]. Diacylglycerol from phosphoinositides is enriched in arachidonate and can serve as a substrate for diacylglycerol lipase and monoacylglycerol lipase to release arachidonate [37]. The rise in intracellular Ca^{2+} which accompanies production of inositol trisphosphate may sustain phospholipase C-mediated hydrolysis of membrane phosphoinositides, or may activate phospholipase A_2 -mediated hydrolysis of more abundant membrane arachidonate-rich phospholipids [38]. The relative importance of each of these pathways in vasoactive-hormone-mediated arachidonate release remains to be determined. The current findings suggest that, in addition, protein kinase C activation, which results from diacylglycerol release, might also be important in augmenting arachidonate release in response to EGF. Indeed, the observed synergistic interaction of EGF with vasopressin supports the potential physiological importance of this mechanism.

Several potential pathways can be postulated for the effect of EGF on arachidonate release and prostaglandin production, and for the synergistic interaction with protein kinase C. In the A431 tumour-cell line, which over-expresses EGF receptors, EGF has been shown to stimulate hydrolysis of phosphatidylinositol bisphosphate and to generate inositol trisphosphate [39] and diacylglycerol [40]. In those studies, EGF was shown to induce a rise in intracellular Ca^{2+} [39]. In mesangial cells, EGF did not increase intracellular Ca^{2+} , and stimulation of prostaglandin production required the presence of the Ca^{2+} ionophore or the Ca^{2+} -mobilizing hormone. These findings are consistent with an effect of EGF to modulate the action of phospholipase A_2 , an enzyme which requires a rise in intracellular Ca^{2+} for activity [38]. Aoyagi *et al.* [9] reported synergism between A23187 and EGF in releasing arachidonate from phosphatidylcholine, suggesting the activation of phospholipase A_2 . Alternatively, EGF could stimulate phospholipase C-mediated breakdown of membrane phospholipids other than phosphatidylinositol bisphosphate to release diacylglycerol as a source of arachidonate without inositol trisphosphate [41].

A potential mechanism for EGF to modulate phospholipase A_2 activity involves the phosphorylation of lipocortin [5]. These proteins have been shown to be substrates for growth-factor-mediated tyrosine phosphorylation [3,4], and it has been suggested that phosphorylation of lipocortin blocks its ability to inhibit arachidonate release [42]. Regulation at the level of lipocortin phosphorylation thus seems to provide an attractive site for the effect of EGF on arachidonate release. However, more recently, studies by Davidson and co-workers [43] have shown that lipocortins inhibit arachidonate release *in vitro* by binding to phospholipid substrate, thus questioning the physiological importance of its modulatory role in prostaglandin production. Therefore it remains to be determined whether this effect of EGF on the mesangial cell involves lipocortin.

The findings of the current study identify a rapid and pronounced effect of EGF on arachidonate release and PGE_2 production which is synergistic with, but not dependent on, activation of protein kinase C. This response may eventually allow delineation of the specific

pathways involved in EGF action. In addition, these findings are of relevance with regard to the regulation of renal prostaglandin production in physiological and disease states. In immune renal disease, prostaglandins have important functions in modulating glomerular filtration rate and inflammation [44]. It is in these states that local EGF concentrations may be the highest [45]. Greater appreciation of the short-term effects of EGF on arachidonate release and its interaction with vasoactive hormones may delineate additional physiological roles for this hormone *in vivo*.

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REFERENCES

- Schlessinger, J., Schreiber, A. B., Levi, A., Lax, J., Lieberman, T. & Yarden, Y. (1983) *CRC Crit. Rev. Biochem.* **14**, 93–111
- Hunter, T. & Cooper, J. A. (1985) *Annu. Rev. Biochem.* **54**, 897–930
- Pepinsky, R. B. & Sinclair, L. K. (1986) *Nature (London)* **321**, 81–84
- Fava, R. A. & Cohen, S. (1984) *J. Biol. Chem.* **259**, 2636–2645
- Hirata, F. (1984) *Adv. Inflammation Res.* **7**, 71–78
- Levine, L. & Hassid, A. (1977) *Biochem. Biophys. Res. Commun.* **76**, 1181–1187
- Tashjian, A. H. & Levine, L. (1978) *Biochem. Biophys. Res. Commun.* **85**, 966–975
- Hirata, Y., Uchihashi, M., Nakoshima, H., Fujita, T., Matsukura, S. & Matsui, K. (1984) *Acta Endocrinol. (Copenhagen)* **107**, 125–130
- Aoyagi, T., Suya, H., Kato, N., Nemoto, O., Kobayashi, H. & Miura, Y. (1985) *J. Invest. Dermatol.* **84**, 168–171
- Yokota, K., Kusaka, M., Oshima, T., Yamamoto, S., Kurihara, N., Yoshino, T. & Kumegawa, M. (1986) *J. Biol. Chem.* **261**, 15410–15415
- Schlondorff, D. (1986) *Am. J. Med.* **81**, Suppl. **2B**, 1–11
- Olsen, P. S., Nexø, E., Poulsen, S. S., Hansen, H. F. & Kirkegaard, P. (1984) *Regul. Pept.* **10**, 37–45
- Rall, L. B., Scott, J., Bell, G. I., Crawford, R. F., Penschow, J. D., Niall, H. D. & Coghlan, J. P. (1985) *Nature (London)* **313**, 228–231
- Scharschmidt, L. A. & Dunn, M. J. (1983) *J. Clin. Invest.* **71**, 1756–1764
- Savage, C. R., Jr & Cohen, S. (1972) *J. Biol. Chem.* **247**, 7609–7611
- Harper, P. A., Robinson, J. M., Hoover, R. L., Wright, T. C. & Karnovsky, M. J. (1984) *Kidney Int.* **26**, 875–880
- Carpenter, G. (1985) *Methods Enzymol.* **109**, 101–110
- Ardailou, N., Hagege, J., Nivez, M., Ardailou, R. & Schlondorff, D. (1985) *Am. J. Physiol.* **248**, F240–F246
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Bonventre, J. V., Skorecki, K. L., Kreisberg, J. I. & Cheung, J. Y. (1986) *Am. J. Physiol.* **251**, F94–F102
- Gelfand, E. W., Cheung, R. K. & Grinstein, S. (1986) *J. Biol. Chem.* **261**, 11520–11523
- Cochet, G., Gill, G. N., Meisenhelder, J., Cooper, J. A. & Hunter, T. (1984) *J. Biol. Chem.* **259**, 2553–2558
- Troyer, D. A., Kreisberg, J. I., Schwartz, D. W. & Venkatachalam, M. A. (1985) *Am. J. Physiol.* **249**, F139–F147
- Williamson, J. R. (1986) *Hypertension* **8**, Suppl. II, II-140–II-156
- Nishizuka, Y. (1986) *Science* **233**, 305–312
- Sinnet-Smith, J. W. & Rozengurt, E. (1985) *J. Cell. Physiol.* **124**, 81–86
- Kreisberg, J. I. (1983) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42**, 3053–3057
- Schreiner, G. F., Kiely, J.-M., Catran, R. S. & Unanue, E. R. (1981) *J. Clin. Invest.* **68**, 920–931
- Lovett, D. H., Sterzel, R. B., Kashgarian, M. & Ryan, J. L. (1983) *Kidney Int.* **23**, 342–349
- Stoscheck, C. M. & King, L. E. (1986) *J. Cell. Biochem.* **31**, 135–152
- Rozengurt, E. (1986) *Science* **234**, 161–166
- Lechner, J. F. & Kaighn, M. E. (1980) *Cell Biol. Int. Rep.* **4**, 23–28
- King, A. C. & Cuatrecasas, P. (1982) *J. Biol. Chem.* **257**, 3053–3060
- Bjorge, J. D. & Kudlow, J. E. (1987) *J. Biol. Chem.* **262**, 6615–6622
- Levine, L., Xiao, D., Worth, N. & Lilley, W. E. (1985) *Biochem. Biophys. Res. Commun.* **130**, 110–117
- Schlondorff, D., Perez, J. & Satriano, J. A. (1985) *Am. J. Physiol.* **248**, C119–C126
- Majerus, P. W., Connolly, T. M., Deckmyn, H., Ross, T. S., Bross, T. E., Ishii, H., Bensal, V. S. & Wilson, D. B. (1986) *Science* **234**, 1519–1526
- Abdel-Latif, A. A. (1986) *Pharmacol. Rev.* **38**, 227–272
- Hepler, J. R., Nakahata, N., Lovenberg, T. W., DeGuiseppe, J., Herman, B., Earp, H. S. & Harden, T. K. (1987) *J. Biol. Chem.* **262**, 2951–2956
- Whiteley, B. & Glaser, L. (1986) *J. Cell Biol.* **103**, 1355–1362
- Besterman, J. M., Duronio, V. & Cuatrecasas, P. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6785–6789
- Touqui, L., Rothhut, B., Shaw, A., Fradin, A., Vargaftig, B. B. & Russo-Marie, F. (1986) *Nature (London)* **321**, 177–180
- Davidson, F. F., Dennis, E. A., Powell, M. & Glenney, J. R., Jr. (1987) *J. Biol. Chem.* **262**, 1698–1705
- Stork, J. E., Rahman, M. A. & Dunn, M. J. (1986) *Am. J. Med.* **80**, Suppl. **1A**, 34–45
- Goodyer, P. R., Kachra, Z., Goodyer, C. & Kaplan, B. (1987) *Kidney Int.* **31**, 167a

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