*Kidney International, Vol. 42 (1992), pp. 559–566*

# Cytokine regulation of adenylate cyclase activity in LLC-PK$_1$ cells

ROBERT J. ANDERSON and RUTH BRECKON

*Department of Medicine, Denver Veterans Affairs Medical Center, and University of Colorado Health Sciences Center, Denver, Colorado, USA*

**Cytokine regulation of adenylate cyclase activity in LLC-PK$_1$ cells.** Although several cytokines have been demonstrated to exert pleiotropic responses, there is little information on cytokine regulation of renal tubular epithelial cell function. In the present studies, we find that both T cell-derived (tumor necrosis factor-$\beta$ and interleukins 2 and 3) and monocyte/macrophage derived (tumor necrosis factor alpha and interleukin 1$\beta$) cytokines promote basal, arginine vasopressin- and forskolin-stimulated adenylate cyclase activity in cultured LLC-PK$_1$ cells. No effect of TNF, IL-1$\beta$, and IL-2 to stimulate protein kinase C activity was observed. TNF-$\beta$, IL-1$\beta$ and IL-2 also modestly stimulated $^3$H release from $^3$H-arachidonic acid labeled cells. Mepacrine, a phospholipase A inhibitor, prevented TNF-$\beta$ stimulation of $^3$H release from $^3$H-arachidonic acid labeled cells and TNF-$\beta$ potentiation of adenylate cyclase activity. TNF-$\beta$ potentiation of adenylate cyclase activity and stimulation of $^3$H release from $^3$H arachidonic acid labeled cells was not prevented by pertussis toxin. These results demonstrate that several cytokines can stimulate adenylate cyclase activity while not affecting protein kinase C activity in cultured renal tubular epithelial cells. The effect of TNF-$\beta$ to stimulate adenylate cyclase appears to occur independent of pertussis toxin-sensitive substrate and may involve activation of phospholipase A.

The term cytokine refers to a large group of soluble molecules, generally glycoproteins, that are produced by activated T lymphocytes, monocytes, macrophages, fibroblasts and stromal cells [1]. Initially, cytokines were thought to mediate immune and inflammatory responses [1]. More recently it has become recognized that cytokines are pleiotropic with multiple biologic activities [1].

With regard to the kidney, several cytokines have been found to exert a variety of actions in glomerular mesangial cells [2–9]. There is, however, limited information on the effect of cytokines on renal tubular epithelial cells [10–12]. Moreover, the signal transduction pathways by which cytokines induce their biological effects remain unclear [1–13]. The present studies were therefore undertaken to examine the effect of both T cell-derived (tumor necrosis factor-beta and interleukins 2 and 3) and macrophage/monocyte-derived (tumor necrosis factor-alpha and interleukin 1) cytokines on signal transduction in cultured renal tubular epithelial cells. Our results demonstrate that multiple cytokines promote adenylate cyclase activity while not affecting protein kinase C activity in LLC-PK$_1$ cells.

Received for publication January 23, 1992
and in revised form April 17, 1992
Accepted for publication April 20, 1992



## Methods

### *Materials*

Arginine vasopressin (AVP), indomethacin, mepacrine, 4-$\beta$-phorbol 12-myristate 13-acetate (PMA), prostaglandin $E_2$ ($PGE_2$) and prostacyclin were obtained from Sigma. Phosphatidylserine and diolein were obtained from Avanti Polar Lipids. Forskolin was obtained from Calbiochem and pertussis toxin from List. Cayman was the source of 11-deoxy prostaglandin $E_1$. Human recombinant TNF-$\alpha$, TNF-$\beta$, interleukin 1 beta (IL-1$\beta$) were obtained from R and D Systems. Human recombinant interleukin 2 (IL-2) and interleukin 3 (IL-3) were provided by Dr. Steven Gillis of Immunex Research and Development Corporation. [$\alpha$-$^{32}$P]ATP (30 to 40 Ci/mmol), [gamma-$^{32}$P] ATP (25 to 35 Ci/mmol) and $^3$H-arachidonic acid (70 to 80 Ci/mmol) were obtained from DuPont-New England Nuclear (Boston, Massachusetts, USA). Amersham was the source of the $PGE_2$ radioimmunoassay kit.

### *Cell and culture conditions*

LLC-PK$_1$ cells were obtained from the American Type Culture Collection. Membranes for adenylate cyclase assays were obtained from cells grown on 75-cm$^2$ plastic flasks. Cells were grown in RPMI 1640 medium (Irvine Scientific) which was supplemented with 200 mg% sodium bicarbonate, 16 mM Hepes, 100 U/ml penicillin, and 200 $\mu$g/ml streptomycin. Newborn calf serum (6%) was added to the media for the initial three to seven days of growth while monolayer proliferation was established after which all cultures received serum-free media. Serum-free conditions were used for 48 hours prior to each experiment. The cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$. Cell growth was monitored under an inverted microscope with phase optics (Nikon IM35) and typically reached confluence in five to seven days. Confluent cultures were selected for biochemical studies.

### *Crude membrane preparation*

Confluent cultures were washed three times with 8 ml of cold phosphate-buffered saline containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ and the cells harvested by scraping three times with 4 ml

of ice-cold EDTA (1 mM)/Tris (50 mM) buffer (pH 7.4). Membranes were homogenized with 15 strokes of a Dounce homogenizer, centrifuged at 14,000 rpm at 4°C for five minutes, and resuspended in 500 μl of EDTA/Tris buffer.

### *Adenylate cyclase assay*

Adenylate cyclase activity was measured using slight modifications of previously described techniques and carried out in a final volume of 50 μl [14–16]. Ten μl of membrane preparation (approximately 10 μg of protein) was added to an assay mixture containing 0.25 mM $Na_2EDTA$, 5.25 mM $MgCl_2$, 1.0 mM cAMP, 1.0 mM purified [$\alpha$-$^{32}$P]ATP ($2 \times 10^6$ cpm) in 25 mM Tris-HCl (pH 7.4) and an ATP-regenerating system consisting of 20 mM creatinine phosphate and 100 IU/ml creatinine phosphokinase. After a 10 minute incubation at 30°C, the reaction was stopped by adding 150 μl of ice-cold stopping solution containing 3.3 mM ATP, 4 mM cAMP, 40 mM Tris-HCl, and 10,000 to 15,000 cpm of [$^3$H]cAMP for recovery, which averaged 50 to 70%. [$^{32}$P]cAMP was separated from unreacted [$\alpha$-$^{32}$P]ATP by a sequential two-step elution over Dowex and alumina columns. Assays were carried out in triplicate with a standard error of the mean usually less than 2 to 3%. Either preliminary experiments were done to document that the vehicles used for the tested cytokines did not affect adenylate cyclase activity or the vehicles were used in control experiments.

### *Protein kinase C assay*

Cells were grown to confluence as described above and protein kinase C activity in the cytosol and particulate fractions measured as previously described [15, 16]. After exposure to test agents, the cultures were washed with ice-cold PBS, scraped into 1 ml of homogenizing buffer (20 mM Tris, 2 mM EGTA, 50 mM 2-mercaptoethanol, pH 7.5) and sonicated for 2 × 15 seconds at the minimum output of a Branson sonicator. The sonicate was centrifuged at 1,000 g for 10 minutes to pellet cell debris and nuclei, and the supernatant was subjected to ultracentrifugation for 60 minutes at 100,000 g to obtain the cytosolic fraction. The particulate fraction was resuspended in 1 ml of the same buffer containing 0.1% Triton X-100, sonicated as above, and again subjected to centrifugation for 60 minutes at 100,000 g. The supernatant thus obtained was used to assay for protein kinase C contained in a particulate fraction. Protein kinase C was assayed in a reaction mixture containing 5 μmol Tris/HCl, pH 7.5, 50 μg histone, 60 μM phosphatidylserine, 8 μM diolein, 1.25 μmol $MgCl_2$, varying concentrations of $CaCl_2$, and 2.5 nmol ATP containing approximately $10^6$ cpm of [gamma$^{32}$P]ATP in a final volume of 0.25 ml. The trichloroacetic acid-precipitable material was collected by filtration through a 0.45 μm nitrocellulose membrane filter (Millipore) washed extensively and analyzed by liquid scintillation counting. Protein kinase activity was calculated as the stimulated activity determined in the presence of calcium and various activators minus the basal activity determined without calcium or other activators.

### *Arachidonic acid and/or metabolite release assays*

These studies were performed by slight modifications of described techniques [17, 18]. Cells were grown in 35 mm dishes and labeled for 18 hours with 0.33 μCi/ml [$^3$H]-arachidonic acid in RPMI 1640 medium (Irvine Scientific) which was supplemented with 200 mg% sodium bicarbonate, 16 mM Hepes and 0.5% fetal calf serum. At the end of the labeling period, cells were washed four times with RPMI and 0.5% fetal calf serum. One ml of RPMI containing 0.5% fetal calf serum with or without the test agent was added and the cells were incubated at

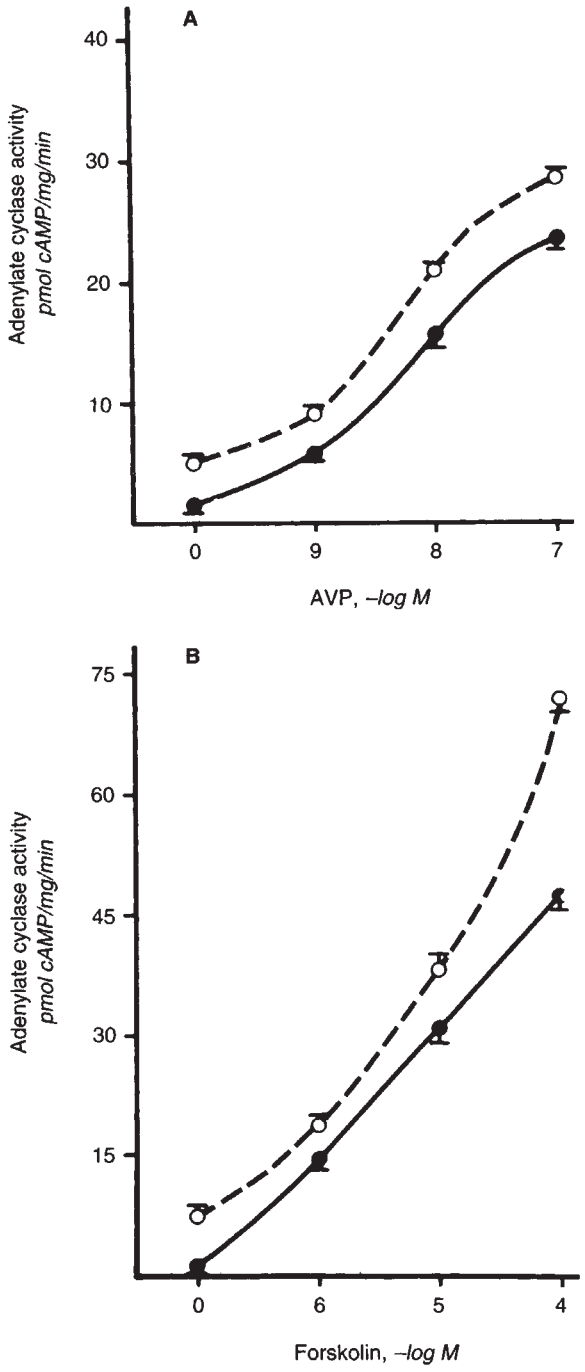


**Fig. 1.** *Effect of TNF-β ($10^{-10}$ M) on AVP- (A) and forskolin- (B) stimulated adenylate cyclase activity.* Symbols are: (○----○) TNF-β; (●——●) control. LLC-$PK_1$ cells were exposed to TNF-β for 5 minutes, cell membranes prepared and adenylate cyclase activity determined as described in *Procedures*. Each data point represents the mean ± SEM of 4 separate experiments done in triplicate. TNF-β significantly increased adenylate cyclase activity at each concentration of agonist.

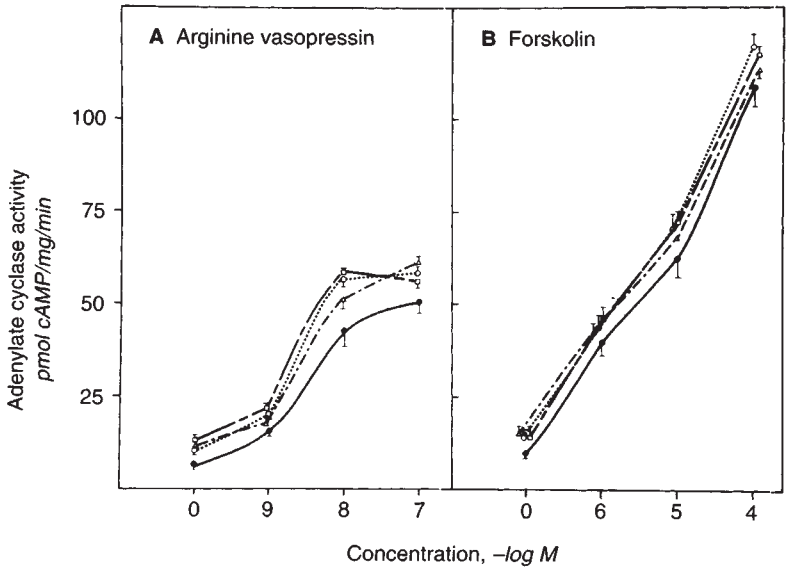


**Fig. 2.** *Effect of control (●——●) $10^{-10}$ M IL-1β (○----○), IL-2 (□——□) and IL-3 (△——△) on AVP- (A) and forskolin- (B) stimulated adenylate cyclase activity in LLC-PK$_1$ cells.* Intact LLC-PK$_1$ cells were exposed to the indicated cytokine for 5 minutes, cell membrane prepared and adenylate cyclase measured as described in *Procedures*. Each data point represents the mean ± SEM of 3 separate experiments done in triplicate. Each of the tested cytokines significantly ($P < 0.05$) increased adenylate cyclase activity when compared with control values at each concentration of agonist, except for IL-3 which did not significantly increase $10^{-4}$ M forskolin-stimulated adenylate cyclase activity.

37°C for the indicated period. The medium was aspirated and counted in a liquid scintillation counter. Three wells were studied under each condition of the assay and the values were averaged to obtain an $N$ of 1 for that condition. The values are expressed as either actual dpm (experimental-control) or percent dpm increase relative to simultaneously studied controls.

### *Prostaglandin $E_2$ assay*

Cells were grown to confluence in 24 well dishes using RPMI media with 6% fetal calf serum. For 12 to 24 hours prior to assay, the cells were exposed to 2% fetal calf serum. Cells were washed three times with Krebs-Ringer solution containing 1 mM calcium. Control cells and experimental cells were incubated for 30 to 60 minutes after which the fluid was removed from each well into polypropylene tubes and frozen at −70°C. $PGE_2$ was measured within one to three days using an Amersham kit (#RPA539).

### *Protein assay*

Protein was estimated by a modified Lowry method using bovine serum albumin as a standard.

### *Statistical analyses*

All calculations and analyses were carried out using at ATT PC-6300 desktop computer and ABSTAT software. All data were expressed at the mean ± SE. Statistical analyses were performed using paired and unpaired Student's $t$-test and ANOVA where appropriate. A $P$ value of <0.05 was considered significant.

## Results

In preliminary dose-response experiments, we found that TNF-β reproducibly potentiated basal and agonist-stimulated adenylate cyclase activity at threshold concentrations ranging from $10^{-12}$ to $10^{-11}$ M. Maximal stimulation occurred at $10^{-10}$ M. In preliminary time course experiments, we found that exposure of intact LLC-PK$_1$ cells to $10^{-10}$ M TNF-β for one, five and 15 minutes produced a comparable increase in cell membrane adenylate cyclase activity. The effect of five minutes exposure to $10^{-10}$ M TNF-β on basal, AVP- and forskolin-stimulated adenylate cyclase activity is in Figure 1. TNF-β significantly ($P < 0.05$) increased basal, AVP- and forskolin (all concentrations tested)-stimulated adenylate cyclase activity. Comparable stimulation of basal, AVP- and forskolin-stimulated adenylate cyclase activity was also seen with $10^{-10}$ M TNFα (data not shown). TNF-β also significantly increased AVP-stimulated adenylate cyclase activity when added directly to LLC-PK$_1$ crude membranes. In these paired studies, adenylate cyclase activity (pmol cAMP/mg/min) stimulated by $10^{-9}$, $10^{-8}$ and $10^{-7}$ M AVP was significantly higher ($P < 0.05$) in the presence than in the absence of TNF-β ($10^{-9}$ M) and was 39.7 ± 2.0 versus 33.8 ± 2.1, 74.8 ± 3.9 versus 65.1 ± 4.2 and 83.8 ± 4.9 versus 70.2 ± 4.0, respectively.

To determine if the effect of TNF to stimulate adenylate cyclase activity was specific for this cytokine, additional studies were performed. Human recombinant IL-1β, IL-2 and IL-3 also significantly ($P < 0.05$) potentiated basal, AVP- and forskolin-stimulated adenylate cyclase activity (Fig. 2).

Since activation of protein kinase C promotes adenylate cyclase activity in LLC-PK$_1$ cells [16], the effect of five minutes exposure to TNF-β on LLC-PK$_1$ cell protein kinase C activity was measured (Fig. 3). The phorbol ester PMA ($10^{-7}$ M) significantly increased protein kinase C specific activity and translocated this activity from a soluble to a particulate cell fraction. By contrast, TNF-β (Fig. 3) did not affect either total activity or cellular localization of protein kinase C. In additional studies, none of the other cytokines tested including TNFα (control 0.660 ± 0.16, $10^{-10}$ M TNFα 0.579 ± 0.09 and $10^{-9}$ M TNFα 0.631 ± 0.12, nmol $^{32}$P/mg/min, $N = 5$), IL-1-β (control 0.801 ± 0.11, $10^{-10}$ M IL-1β 0.718 ± 0.06 and $10^{-9}$ M IL-1β 0.759 ± 0.10 nmol $^{32}$P/mg/min, $N = 5$) and IL-2 (control 0.540 ± 0.10, $10^{-10}$ M IL-2 0.434 ± 0.08 and $10^{-9}$ M IL-2 0.408 ± 0.08 nmol $^{32}$P/mg/min, $N = 5$) significantly affected specific activity or localization of protein kinase C activity. Together these results suggest that the effect of TNF, IL-1β and IL-2 to

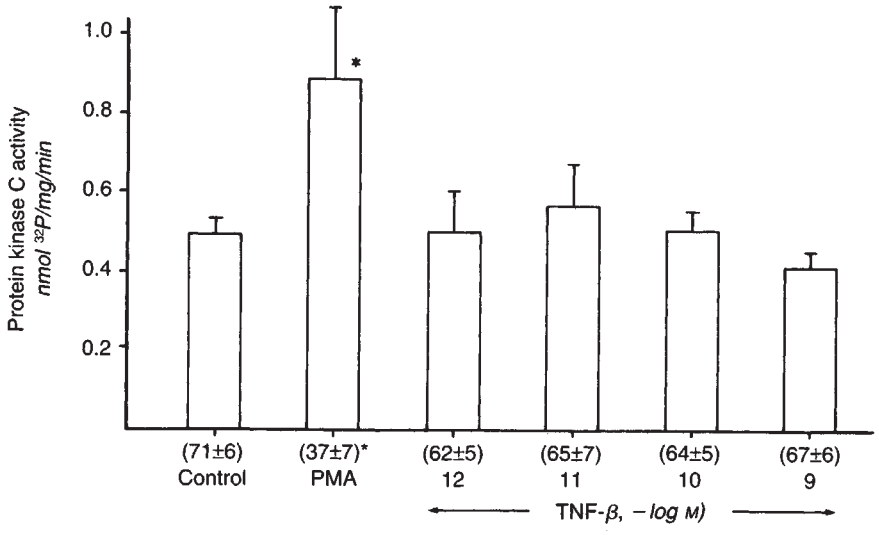


**Fig. 3.** *Effect of TNF-β and PMA on protein kinase C activity in LLC-PK$_1$ cells.* Intact cells were exposed for 5 minutes to the noted concentrations of TNF-β or to $10^{-7}$ M PMA. Cell fractions were obtained and analyzed for protein kinase C as described in *Procedures*. Each bar represents the mean ± SEM of 5 experiments. The number denoted in parentheses at the bottom of each column represents the percentage of protein kinase C found in a particulate fraction. * $P < 0.05$ when compared with control.

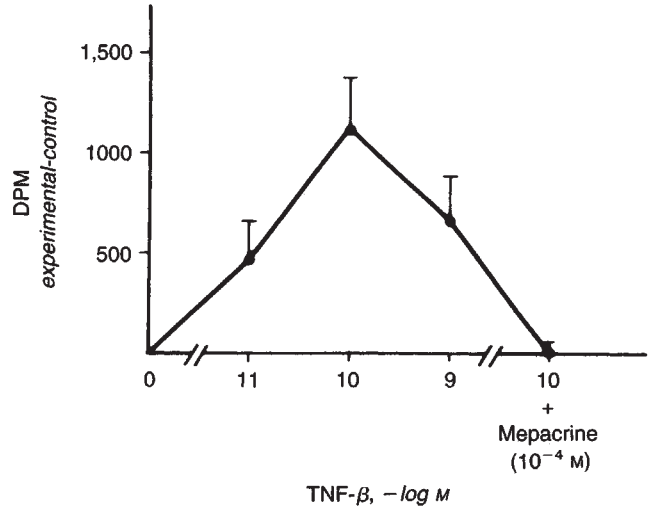


**Fig. 4.** *Effect of TNF-β on $^3$H release from $^3$H-arachidonic acid labeled LLC-PK$_1$ cells.* Intact LLC-PK$_1$ cells were exposed to the noted concentrations of TNF in the absence or presence of mepacrine for 5 minutes. The individual data points represent the mean ± SEM of experimental control DPM found in the media. At $10^{-10}$ M, TNF-β significantly increases DPM and this effect was abolished in the presence of mepacrine pretreatment (30 min).

stimulate adenylate cyclase activity occurs independent of protein kinase C activity.

TNF activates phospholipase A in several cell types [2, 4, 6, 10, 13, 19–21]. To indirectly assess if TNF-β activates phospholipase A in LLC-PK$_1$ cells, intact cells were incubated for 18 hours with $^3$H-arachidonic acid then exposed for five minutes to several cytokines. The effect of TNF-β on $^3$H release is in Figure 4. TNF-β resulted in a modest, significant release of $^3$H from $^3$H-arachidonic acid labeled cells. This release of $^3$H was 65 ± 9% greater than observed in control cells not exposed to TNF-β studied over the same time interval. This release of $^3$H could be completely prevented by pre-treatment with $10^{-4}$ M mepacrine, a phospholipase A inhibitor. We also examined the effect of IL-1β and IL-2 on $^3$H release from $^3$H arachidonic acid labeled cells. At $10^{-10}$ M, IL-1β stimulated release of $^3$H from labeled cells at a rate of 13 ± 4% greater than control cells ($N$ = 4) while $10^{-10}$ M IL-2 stimulated $^3$H release at a rate of 49 ± 10% greater than control cells ($N$ = 5). Pretreatment with $10^{-4}$ M mepacrine completely prevented any increase in $^3$H release from $^3$H-arachidonic acid labeled cells with either $10^{-10}$ M IL-1β or IL-2 relative to control cells. These observations suggest that TNF-β, IL-1β and IL-2 stimulate release of arachidonic acid and/or its metabolites in LLC-PK$_1$ cells and this release is blocked by mepacrine.

To determine the role of phospholipase A activation in TNF potentiation of adenylate cyclase activity, the paired experiments depicted in Figure 5 were done. At $10^{-10}$ M, TNF-β stimulation of adenylate cyclase occurred in the absence but not in the presence of phospholipase A inhibition with mepacrine, suggesting the possibility that either arachidonic acid or one of its metabolic products was responsible for TNF-β potentiation of adenylate cyclase activity.

Arachidonic acid can be metabolized via cyclooxygenase to prostaglandins, prostacyclin and thromboxanes, via lipooxygenase to hydroperoxyeicosatetraenoic acid and leukotrienes and via cytochrome P450 monooxygenase to epoxyeicostraenoic acids. The two major cyclooxygenase products produced by most renal tubular epithelial cells are prostaglandin $E_2$ and prostacyclin. Since prostaglandins and prostacyclin can regulate adenylate cyclase activity in several cell types [22, 23], we determined if $PGE_2$ and prostacyclin are candidate effectors for TNF-β potentiation of adenylate cyclase activity in LLC-PK$_1$ cells. At $10^{-11}$ M, $PGE_2$ did not affect AVP-stimulated adenylate cyclase activity. At $10^{-10}$, $10^{-9}$ and $10^{-8}$ M, $PGE_2$ potentiated AVP-stimulated adenylate cyclase activity by 8 ± 3, 12 ± 2 and 27 ± 4%, respectively. The effect of $10^{-8}$ M $PGE_2$ on AVP-stimulated adenylate cyclase activity is depicted in Figure 6. To clarify the prostaglandin receptor subtype involved in $PGE_2$ potentiation of adenylate cyclase activity [24], the effect of 11-deoxy prostaglandin $E_1$ was measured (Table 1). The effect of both prostaglandin $E_2$ (Fig. 6) and 11-deoxy prostaglandin $E_1$ (Table 1) to potentiate AVP-stimulated adenylate cyclase activity suggest participation of the $EP_2$ subtype of

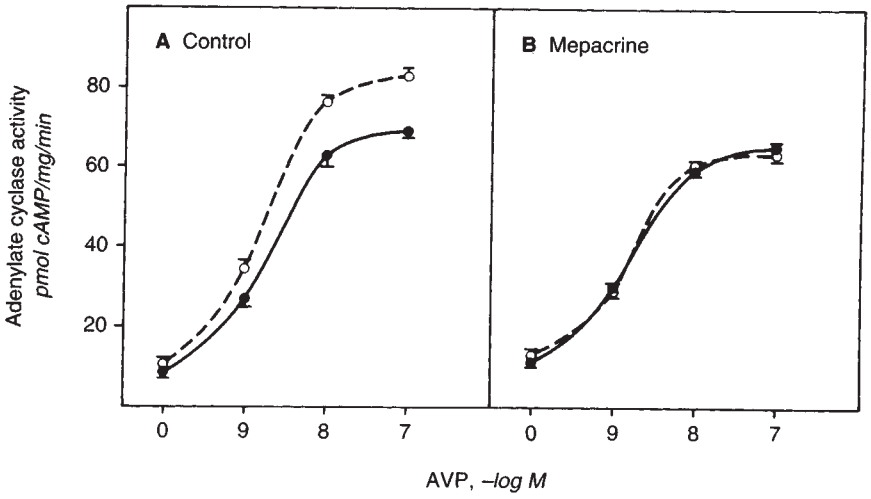


**Fig. 5.** *Effect of mepacrine pretreatment on TNF-β potentiation of AVP-stimulated adenylate cyclase activity in LLC-PK$_1$ cells.* Symbols are (○) TNF-β; (●) control. Intact LLC-PK$_1$ were pre-treated (30 min) with $10^{-4}$ M mepacrine (**B**) or mepacrine carrier solution (control, **A**). Intact cells were then exposed to the noted concentrations of AVP in the presence and absence of $10^{-10}$ M TNF-β for 5 minutes. Cell membranes were obtained and analyzed for adenylate cyclase activity as noted in *Procedures*. Each data point represents the mean ± SEM of 4 paired experiments performed in triplicate. TNF-β significantly ($P < 0.05$) increased adenylate cyclase activity at all AVP concentrations in the absence, but not in the presence of mepacrine.

prostaglandin receptor in this process [24]. As noted in Table 1, $10^{-8}$ M prostacyclin also modestly potentiates AVP-stimulated adenylate cyclase activity as noted in other studies in renal epithelial cells [22, 23].

Since our results suggest that cyclooxygenase products of arachidonic acid are possible effectors for TNF potentiation of adenylate cyclase activity, additional experiments were performed. First, we attempted to determine if TNF stimulates release of $PGE_2$ from LLC-PK$_1$ cells. In these paired studies ($N = 9$) media from control and TNF-β-treated cells ($10^{-10}$ M for 30 min) was assayed for $PGE_2$ using radioimmunoassay. We found very low levels of $PGE_2$ in control cells (3.66 ± 0.17 pg/μg protein/30 min) that did not significantly increase with TNF treatment (3.70 ± 0.32 pg/μg/protein/30 min). Since it is possible that our radioimmunoassay, under the conditions of the experiment, was insufficiently sensitive to detect TNF-β-stimulated $PGE_2$ production, pharmacologic experiments were performed. In these studies, we examined the effect of indomethacin pre-treatment ($10^{-5}$ M) on basal and AVP-stimulated adenylate cyclase activity in the presence and absence of $10^{-10}$ M TNF-β (Table 2). Indomethacin prevented TNF-β potentiation of AVP-stimulated but not basal adenylate cyclase activity. To determine if lipooxygenase metabolites could play a role in mediating TNF-β potentiation of AVP-stimulated adenylate cyclase activity, preliminary studies were undertaken with nordihydroguaiaretic acid (NGDA, $10^{-4}$ M). NGDA prevented $10^{-9}$ M TNF-β to potentiate $10^{-7}$ AVP-stimulated adenylate cyclase activity (41.3 ± 4.1 to 41.1 ± 3.6 in the presence and 44.7 ± 3.5 to 62.1 ± 8.4 pmol cAMP/mg/min in the absence of NGDA).

G proteins are known to regulate adenylate cyclase activity, and several reports suggest a role for G proteins, including a pertussis toxin-sensitive G protein, in receptor-stimulated activation of phospholipase A [25–27]. To determine if TNF-β activation of phospholipase A and potentiation of adenylate cyclase activity involves a pertussis toxin-sensitive G protein, two types of studies were done. First, we examined the effect of $10^{-10}$ M TNF-β to stimulate $^3H$ release from $^3H$-arachidonic acid labeled in the absence and presence of pertussis toxin (1.0 μg/ml for 18 hr). In five paired experiments, TNF increased $^3H$ release from $^3H$-arachidonic acid labeled cells by 34 ± 4% in the absence and 44 ± 5% in the presence of pertussis toxin pretreatment. In a second set of experiments, we examined the effect of pertussis toxin pretreatment on TNF-β potentiation of adenylate cyclase activity (Fig. 7). TNF-β potentiated adenylate cyclase activity in both the presence and absence of pertussis toxin.

## Discussion

In the present studies we find that cytokines of diverse origin including TNF, IL-1β, IL-2 and IL-3 promote adenylate cyclase activity in a cultured renal tubular epithelial cell line. This stimulation occurs under basal conditions as well as in the presence of a hormonal (AVP) and a direct (forskolin) activator of adenylate cyclase. Our studies in crude membranes also suggest that the effect of TNF-β to potentiate adenylate cyclase activity occurs exclusively within the cell membrane compartment. To our knowledge, an effect of any cytokine to stimulate adenylate cyclase activity has not been previously reported. Indirect observations support the contention that selected cytokines are capable of stimulating adenylate cyclase activity in several cell types. For example, TNFα is a potent stimulator of cAMP accumulation in human fibroblasts and rat mesangial cells [2, 28, 29]. Although these results are compatible with an effect of TNFα to stimulate adenylate cyclase activity, TNFα has also been reported to inhibit agonist stimulated cAMP accumulation in other cell types [30–33]. With regard to other cytokines, IL-1 stimulates cAMP accumulation in most [28, 29, 34, 35] but not all studies [33]. Finally, in the only study directly examining the effect of a cytokine on adenylate cyclase activity, IL-2 has been reported to exert a protein kinase C-dependent inhibition of adenylate cyclase activity in murine lymphocytes [36]. Taken together, the results of the present and previous studies suggest that several cytokines can stimulate adenylate cyclase activity, thereby increasing cAMP and protein kinase A in selected cell types. It is therefore possible that activation of this signal transduction pathway may underlie some of the physiologic response to cytokines [35].

Activation of protein kinase C can stimulate adenylate cyclase activity in LLC-PK$_1$ cells [16]. In selected cells, some cytokines have been reported to stimulate protein kinase C activity [36, 37]. While it appears clear that serine and threonine

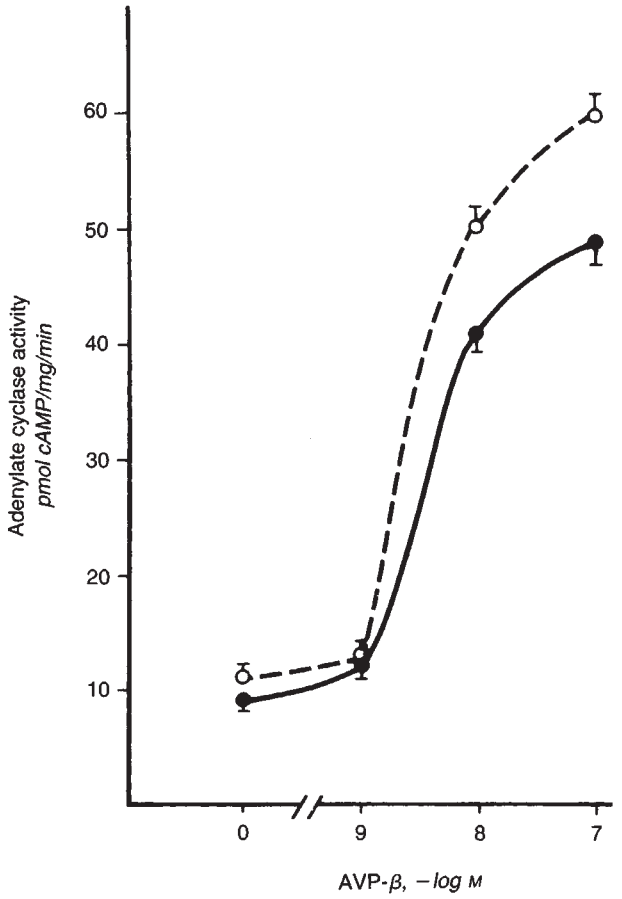


**Fig. 6.** *Effect of prostaglandin $E_2$ on AVP-stimulated adenylate cyclase activity in LLC-PK$_1$ cells.* Symbols are: (○----○) $PGE_2$; (●——●) control. Intact cells were exposed for 5 minutes to $10^{-8}$ M $PGE_2$. Cell membranes were obtained and adenylate cyclase assayed as described in *Procedures*. Each data point represents the mean ± SEM of four experiments performed in triplicate. $PGE_2$ significantly ($P < 0.05$) increased adenylate cyclase activity under each condition except in the presence of $10^{-9}$ M AVP.

**Table 1.** Effect of prostacyclin and 11-deoxy prostaglandin $E_1$ on basal and AVP-stimulated adenylate cyclase activity

| | Adenylate cyclase activity *pmol cAMP/mg/min* | | |
|---|---|---|---|
| Condition | Control | Prostacyclin | 11 Deoxy $PGE_1$ |
| Basal | 3.6 ± 0.3 | 3.2 ± 0.7 | 3.6 ± 0.8 |
| AVP *M* | | | |
| $10^{-9}$ | 10.0 ± 0.9 | 12.8 ± 1.7[a] | 13.3 ± 0.5[a] |
| $10^{-8}$ | 40.9 ± 1.6 | 46.3 ± 2.3[a] | 47.3 ± 1.3[a] |
| $10^{-7}$ | 45.6 ± 1.9 | 53.3 ± 2.8[a] | 57.6 ± 3.4[a] |

LLC-$PK_1$ cells were exposed to nothing (control), prostacyclin ($10^{-8}$ M) or 11-deoxy prostaglandin $E_1$ ($10^{-8}$ M). Cells were harvested, membranes obtained and adenylate cyclase assays performed as described in the presence of the noted concentrations of AVP.
[a] $P < 0.05$ when compared with control

**Table 2.** Effect of TNF-β on arginine vasopressin-stimulated adenylate cyclase activity in the presence and absence of indomethacin

| | Adenylate cyclase activity *pmol cAMP/mg/min* | | | |
|---|---|---|---|---|
| | Indomethacin (−) | | Indomethacin (+) | |
| AVP M | TNF (−) | TNF (+) | TNF (−) | TNF (+) |
| 0 | 6.7 ± 1.1 | 9.1 ± 1.6[a] | 8.2 ± 1.5 | 11.1 ± 1.0[a] |
| $10^{-8}$ | 48.7 ± 3.3 | 59.4 ± 4.0[a] | 51.2 ± 3.4 | 49.2 ± 4.1 |
| $10^{-7}$ | 51.9 ± 3.5 | 62.9 ± 3.7[a] | 56.1 ± 3.8 | 55.5 ± 3.6 |

LLC-$PK_1$ cells were pretreated for 20 minutes with $10^{-5}$ M indomethacin where indicated. Cells were then exposed to either TNF-β carrier solution or to TNF-β ($10^{-10}$ M) for 5 minutes. Cells were harvested, membranes obtained and adenylate cyclase assays performed as described in the presence of the noted concentrations of AVP.
[a] $P < 0.05$ when compared with control

phosphorylation occurs in response to tumor necrosis factor and IL-1 in some cells [3, 29], most studies have been unable to detect an effect of cytokines to stimulate phosphoinositol turnover, increase intracellular calcium concentration or activate protein kinase C [29, 39–41]. Our results are compatible with these latter observations and suggest that the effect of TNF, IL-1 and IL-2 to stimulate adenylate cyclase activity occurs independent of protein kinase C. It is noteworthy that recent studies performed in renal glomerular mesangial cells suggest that IL-1 activates enzymes that increase phosphatidic acid formation [5, 8]. In addition, phosphatidylinositol kinases that potentially result in formation of phosphorylated derivatives of phosphotidylinositol have been described in response to IL-1 and IL-2 in fibroblasts and monocytes [41, 42]. Together, these observations suggest that phosphatidic acid and novel phosphorylated derivatives of phosphatidylinositol may be important second messengers of the actions of selected cytokines.

Recently, TNF and IL-1 have been shown to increase binding of GTP and GTP analogues to a variety of plasma membranes and to stimulate GTPase activity [8, 43, 44]. In several studies, some of the biologic effects of TNF and IL-1 can be attenuated by pertussis toxin [8, 43, 44]. Together, these observations suggest that a pertussis toxin sensitive G protein couples TNF and IL-1 receptors to a biologic response. Moreover, a pertussis toxin sensitive G protein is well known to be involved in regulation of phospholipase $A_2$ and adenylate cyclase activity [25–27]. We therefore examined the effect of pertussis toxin pre-treatment on TNF-β potentiation of adenylate cyclase and $^3H$ release from $^3H$-arachidonic acid labeled cells. Our results suggest that the effect of TNF-β to promote adenylate cyclase and stimulate $^3H$ release from $^3H$-arachidonic acid labeled cells occurs in the presence of high concentrations of pertussis toxin suggesting an effect independent of a pertussis toxin sensitive G protein.

In many cell types [13, 19–21] including rat mesangial cells [2, 4, 6], rabbit inner medullary cells, and cultures MDCK cells [10–12], IL-1 and TNF appear to activate phospholipase $A_2$ with subsequent generation of products of both cyclooxygenase ($PGE_2$) and lipooxygenase [7, 10–12]. In the present studies, we find TNF-β, IL-1β and IL-2 stimulate $^3H$ release from $^3H$-arachidonic acid labeled cells and that this release can be blocked by mepacrine, a putative phospholipase inhibitor. Moreover, the effect of TNF to stimulate adenylate cyclase activity does not occur in the presence of mepacrine and can be attenuated by indomethacin. Finally, high concentrations of exogenous $PGE_2$ stimulate adenylate cyclase activity in our cells. Together, these observations suggest that the effect of selected cytokines that we observed to stimulate adenylate

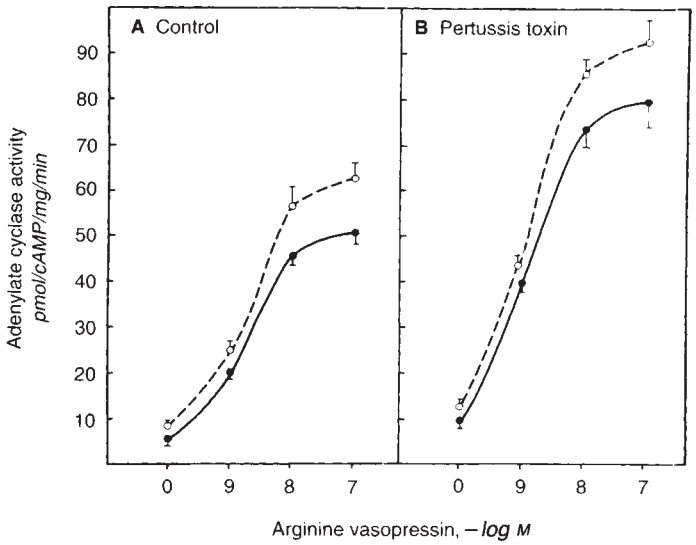


**Fig. 7.** *Effect of pertussis toxin pre-treatment on TNF-β potentiation of adenylate cyclase activity in LLC-PK1 cells.* Symbols are: (○----○) TNF-β; (●——●) control. Intact LLC-$PK_1$ cells were pre-treated (18 hr) with either pertussis toxin (1.0 μg/ml, **B**) or pertussis toxin carrier (control, **A**). Intact cells were then exposed to the noted concentrations of AVP in the presence and absence of $10^{-10}$ M TNF-β for 5 minutes. Cell membranes were obtained and analyzed for adenylate cyclase activity as described under *Procedures*. Each data point represents the mean ± SEM of 4 paired experiments performed in triplicate. TNF-β significantly ($P < 0.05$) potentiated basal and AVP-stimulated adenylate cyclase activity in the presence and absence of pertussis toxin pretreatment.

cyclase may occur, at least in part, as a result of activation of phospholipase $A_2$ and subsequent generation of products of cyclooxygenase. This interpretation of our results however must be tempered by the fact that we could not demonstrate an effect of TNF to stimulate $PGE_2$ production in LLC-$PK_1$ cells. Moreover, other investigators have also found that LLC-$PK_1$ cells produce little if any $PGE_2$ and suggest that these cells have a defect in cyclooxygenase [45, 46]. It is also possible that the high concentration of mepacrine used may exert effects completely unrelated to phospholipase A. Finally, preliminary observations demonstrate that not only inhibition of cyclooxygenase but also inhibition of lipooxygenase can prevent TNF potentiation of adenylate cyclase. Clearly, further studies are required to better identify the mechanism(s) whereby cytokines potentiate adenylate cyclase activity in LLC-$PK_1$ cells.

In summary, our results demonstrate that several cytokines of diverse origin are capable of promoting basal and agonist-stimulated adenylate cyclase activity in an established renal tubular epithelial cell line. This stimulation of adenylate cyclase activity appears to occur independently of either protein kinase C or a pertussis toxin sensitive substrate. Although the effect of cytokines to stimulate adenylate cyclases is modest in magnitude, it is possible that cytokines could potentially modulate cyclic AMP responsive renal tubular transport processes. Physiologic studies will be required to pursue this issue.

## Acknowledgments

The studies reported herein were supported by the Research Service of the Department of Veterans Affairs. The authors would like to thank Joyce Fabel for secretarial support and Dr. Steven Gillis (Immunex) for the gift of IL-2 and IL-3.

*Reprint requests to Dr. Robert J. Anderson, Medical Service (111), Denver VA Medical Center, 1055 Clermont Street, Denver, Colorado 80220, USA*

## References

1. Arai K, Lee F, Miyajima A, Miyatake S, Arai N, Yokola T: Cytokines: Coordinators of immune inflammatory responses. *Ann Rev Biochem* 59:783–836, 1990
2. Baud L, Perez L, Friedlander G, Ardaillou R: Tumor necrosis factor stimulates prostaglandin production and cyclic AMP levels in rat cultured mesangial cells. *FEBS Lett* 239:50–54, 1988
3. Lovett DH, Martin M, Bursten S, Szamel M, Gemsa D, Resch K: Interleukin 1 and the glomerular mesangium III. IL-1 dependent stimulation of mesangial cell protein kinase activity. *Kidney Int* 34:26–35, 1988
4. Pfeilschifter J, Pignat W, Vosbeck K, Macki F: Interleukin 1 and tumor necrosis factor synergistically stimulate prostaglandin synthesis and phospholipase $A_2$ release from rat mesangial cells. *Biochem Biophys Res Comm* 159:385–394, 1989
5. Kester M, Simonson MS, Mene P, Sedor JR: Interleukin 1 generates transmembrane signals from phospholipids through novel pathways in cultured rat mesangial cells. *J Clin Invest* 83:718–723, 1989
6. Nakazato Y, Simonson MS, Herman WH, Konieczkowski M, Sedor JR: Interleukin-$1_\alpha$ stimulates prostaglandin biosynthesis in serum activated mesangial cells by induction of a non-pancreatic (type II) phospholipase $A_2$. *J Biol Chem* 266:14119–14127, 1991
7. Steiner P, Pfeilschifter J, Boeckh C, Radeke H, Otten U: Interleukin-1 and tumor necrosis factor$_\alpha$ synergistically stimulate nerve growth factor synthesis in rat mesangial cells. *Am J Physiol* 261:P792–798, 1991
8. Bursten SL, Harris WE, Bomsztzk K, Lovett D: Interleukin-1 rapidly stimulates lysophosphatidate acyltransferase and phosphatidate phosphorylase activities in human mesangial cells. *J Biol Chem* 266:20732–20743, 1991
9. Ruef C, Budde K, Lacy J, Northemann W, Bauman M, Sterzl RB, Coleman DL: Interleukin 6 is an autocrine growth factor for mesangial cells. *Kidney Int* 38:249–257, 1990
10. Kohan DE: Interleukin-1 regulation of prostaglandin $E_2$ synthesis by the papillary collecting duct. *J Lab Clin Med* 114:717–723, 1989
11. Leighton JD, Pfeilschifter J: Interleukin 1- and tumor necrosis factor- stimulation of prostaglandin $E_2$ synthesis in MDCK cells and potentiation of this effect by cycloheximide. *FEBS Lett* 259: 289–292, 1990
12. Zeidel ML, Brady HR, Kohan DE: Interleukin-1 inhibition of $NA^+$-$K^+$ ATPase in inner medullary collecting duct cells: Role of $PGE_2$. *Am J Physiol* 261:F1013–F1016, 1991
13. Vilcek J, Lee TH: Tumor necrosis factor. *J Biol Chem* 255:7313–7316, 1991
14. Dixon BS, Breckon R, Kaehny M, Dillingham MA, Anderson RJ: Histidine regulation of cyclic AMP metabolism in cultured renal epithelial LLC-$PK_1$ cells. *J Biol Chem* 265:760–766, 1990
15. Anderson RJ, Breckon R, Dixon BS: ATP receptor regulation of adenylate cyclase and protein kinase C activity in cultured renal LLC-$PK_1$ cells. *J Clin Invest* 87:1732–1738, 1991
16. Anderson RJ, Breckon RJ: Cyclic AMP stimulates protein kinase C activity in LLC-$PK_1$ cells. *Am J Physiol* 261:F945–F950, 1991
17. Slivka SR, Insel PA: $\alpha_1$-adrenergic receptor-mediated phosphoinositide hydrolysis and prostaglandin $E_2$ formation in mandindarby canine kidney cells. *J Biol Chem* 262:4200–4207, 1987
18. Slivka SR, Insel PA: Phorbol ester and neomycin dissociate bradykinin receptor-mediated arachidonic acid release and phosphoinositide hydrolysis in mandin-darby kidney cells. *J Biol Chem* 263:14640–14647, 1988
19. Mizel SB, Dayer J-M, Krane SM, Mergenhagen SE: Stimulation of rheumatoid synovial cell collagenase and prostaglandin production by partially purified lymphocyte activating factor (interleukin 1). (abstract) *Proc Natl Acad Sci USA* 78:2474, 1981

20. Chang J, Gilman SC, Lewis AJ: Interleukin-1 activates phospholipase $A_2$ in rabbit chondrocytes: A possible signal role for IL-1 action. *J Immunol* 136:1283–1286, 1986
21. Raz A, Wyche A, Siegel N, Needleman P: Regulation of fibroblast cyclooxygenase synthesis by interleukin-1. *J Biol Chem* 263:3022–3028, 1980
22. Grenier FC, Allen ML, Smith WL: Interrelationships among prostaglandins, vasopressin and cAMP in renal papillary collecting tubule cells in culture. *Prostaglandins* 24:547–565, 1982
23. Veis JH, Dillingham MA, Berl T: Effects of prostacyclin on the cAMP system in cultured rat inner medullary collecting duct cells. *Am J Physiol* 258:F1218–F1223, 1990
24. Armstrong RA, Matthews JS, Jones RL, Wilson NH: Characterisation of $PGE_2$ receptors mediating increased vascular permeability in inflammation. *Adv Prostaglandin Thromboxane Leukot Res* 21:375–378, 1990
25. Teitlebaum I: The epidermal growth factor receptor is coupled to a phospholipase $A_2$-specific pertussis toxin-inhibitable guanine nucleotide-binding regulatory protein in cultured rat inner medullary collecting tubule cells. *J Biol Chem* 265:4218–4222, 1990
26. Burch RM, Luini A, Axelrod J: Phospholipase $A_2$ and phospholipase C are activated by distinct GTP-binding proteins in response to $\alpha_1$-adrenergic stimulation in FRTL5 thyroid cells. *Proc Natl Acad Sci USA* 83:7201–7205, 1986
27. Miller RT: Transmembrane signalling through G proteins. *Kidney Int* 39:421–429, 1991
28. Zhang Y, Len J-X, Yip YK, Vilcek J: Enhancement of cAMP levels and of protein kinase activity by tumor necrosis factor and interleukin 1 in human fibroblasts: Role in the induction of interleukin 6. *Proc Natl Acad Sci USA* 85:6802–6805, 1988
29. Guy GR, Chua SP, Wong NS, Ng SB, Tan YH: Interleukin 1 and tumor necrosis factor activate common multiple protein kinases in human fibroblasts. *J Biol Chem* 266:14343–14352, 1991
30. Andreani CL, Payne DW, Packman JN, Resnick CE, Horwitz A, Adoshi EY: Cytokine-medicated regulation of ovarian function. *J Biol Chem* 266:6761–6766, 1991
31. Nathan C, Sanchez E: Tumor necrosis factor and CD11/CD18 (2) integrins act synergistically to lower cAMP in human neutrophils. *J Cell Biol* 111:2171–2181, 1990
32. Shapiro S, Tatakis DN, Dziak R: Effects of tumor necrosis factor alpha on parathyroid hormone-induced increases in osteoblastic cell cyclic AMP. *Calcif Tissue Int* 46:60–62, 1990
33. Gulick T, Chung MK, Pieper SJ, Lange LG, Schreiner GF: Interleukin 1 and tumor necrosis factor inhibit cardiac myocyte$\beta$-adrenergic responsiveness. *Proc Natl Acad Sci USA* 86:6753–6757, 1989
34. Shirakawa F, Yamashita U, Chedid M, Mizel SB: Cyclic AMP—An intracellular second messenger for interleukin 1. *Proc Natl Acad Sci USA* 85:8201–8205, 1988
35. Turunen JP, Mattila P, Renkonen R: cAMP mediates IL-1-induced lymphocyte penetration through endothelial monolayers. *J Immunol* 145:4192–4197, 1990
36. Beckner SK, Farrar WL: Interleukin 2 modulation of adenylate cyclase. *J Biol Chem* 261:3043–3047, 1986
37. Schutze S, Nottrott S, Pfizenmaier K, Kronke M: Tumor necrosis factor signal transduction. *J Immunol* 144:2604–2608, 1990
38. Charkin E, Ziltener HG, Razin E: Protein kinase C plays an inhibitory role in interleukin 3- and interleukin 4-mediated mast cell proliferation. *J Biol Chem* 265:22109–22116, 1990
39. Shiori-Nakaro K, Kuwas MT: Signal requirement for interleukin-1-dependent interleukin 2 production by a human leukemia-derived HSB-2 subclone. *J Immunol* 139:3321–3329, 1987
40. Abraham RT, Ho SW, Barna TJ, McKean DJ: Transmembrane signaling during interleukin 1-dependent T cell activation. *J Biol Chem* 262:2719–2728, 1987
41. Ballou LR, Barker SC, Postlethwaite A, Kang AE: Interleukin 1 stimulates phosphatidyl-inositol kinase activity in human fibroblasts. *J Clin Invest* 87:299–304, 1991
42. Remillard B, Petrillo R, Maslinski LO, Tsudo M, Strom TB, Cantley L, Varticooski L: Interleukin-2 receptor regulates activation of phosphatidylinositol-3-kinase. *J Biol Chem* 266:14167–14170, 1991
43. Imamura K, Sherman ML, Spriggs D, Kafe D: Effect of tumor necrosis factor on GTP binding and GTPase activity in HL-60 and L927 cells. *J Biol Chem* 263:10247–10253, 1988
44. O'Neill LA, Bird TA, Gearing AJ, Saklatvala J: Interleukin-1 signal transduction. *J Biol Chem* 265:3146–3152, 1990
45. Hassid A: Transport-active renal tubular epithelial cells (MDCK and LLC-$PK_1$) in culture. Prostaglandin biosynthesis and its regulation by peptide hormones and ionophore. *Prostaglandins* 21:985–1001, 1981
46. Lifschitz MD: LLC-PK1 cells derived from pig kidneys have a defect in cyclooxygenase. *J Biol Chem* 257:12611–12615, 1982