Evidence for Paracrine Signaling Between Macrophages and Bovine Adrenal Chromaffin Cell $Ca^{2+}$ Channels

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Currie, Kevin P. M., Zhong Zhou, and Aaron P. Fox. Evidence for paracrine signaling between macrophages and bovine adrenal chromaffin cell $Ca^{2+}$ channels. J. Neurophysiol. 83: 280–287, 2000. The adrenal gland contains resident macrophages, some of which lie adjacent to the catecholamine producing chromaffin cells. Because macrophages release a variety of secretory products, it is possible that paracrine signaling between these two cell types exists. Of particular interest is the potential paracrine modulation of voltage-gated calcium channels ($I_{Ca}$) that are the main calcium influx pathway triggering catecholamine release from chromaffin cells. We report that prostaglandin E$_2$ (PGE$_2$), one of the main signals produced by macrophages, inhibited $I_{Ca}$ in cultured bovine adrenal chromaffin cells. The inhibition is rapid, robust, and voltage dependent; the activation kinetics are slowed and inhibition is largely reversed by a large depolarizing prepulse, suggesting that the inhibition is mediated by a direct G-protein $\beta\gamma$ subunit interaction with the calcium channels. About half of the response to PGE$_2$ was sensitive to pertussis toxin (PTX) incubation, suggesting both PTX-sensitive and -insensitive G proteins were involved. We show that activation of macrophages by endotoxin rapidly (within minutes) releases a signal that inhibits $I_{Ca}$ in chromaffin cells. The inhibition is voltage dependent and partially PTX sensitive. PGE$_2$ is not responsible for this inhibition as blocking cyclooxygenase with ibuprofen did not prevent the production of the inhibitory signal by the macrophages. Nor did blocking the lipoxygenase pathway with nordihydroguaiaretic acid alter production of the inhibitory signal. Our results suggest that macrophages may modulate $I_{Ca}$ and catecholamine secretion by releasing PGE$_2$ and other chemical signal(s).

INTRODUCTION

Interactions between the immune system and the adrenal gland are well documented. For instance, there is elevated catecholamine release by the adrenal medulla during periods of stress and infection due both to increased sympathetic stimulation and peripheral mechanisms (Zhou and Jones 1993). In addition, cytokines stimulate the hypothalamic-pituitary-adrenal axis leading to increased glucocorticoid production by the adrenal cortex; this results in a negative feedback immunosuppressive effect (Buckingham et al. 1996; Turnbull and Rivier 1995). As opposed to these indirect neurogenic effects of the immune system on adrenal function, it is becoming increasingly clear that there are direct paracrine interactions within the adrenal gland itself (Marx et al. 1998; Nussdorfer and Mazocchi 1998). Interest in this field has focused largely on the actions of intra adrenal cytokines, especially on cortical cells. These cytokines are produced both by adrenal cells themselves and by macrophages. Both rat and human adrenal glands have a population of resident macrophages distributed throughout the cortex and medulla (Gonzalez-Hernandez et al. 1994; Schober et al. 1998), some of which lie adjacent to the catecholamine producing chromaffin cells. This makes them ideally situated to participate in paracrine signaling to the chromaffin cells and potentially modulate catecholamine release. Such signaling has been suggested previously as a subpopulation of the resident macrophages contain neurotrophin-4 (NT4) and chromaffin cells express the Trk A receptor that binds NT4 (Schober et al. 1998). Other studies have demonstrated that a peptide released by human monocytes stimulated catecholamine release from cultured adrenal chromaffin cells (Jones et al. 1993; Roberts et al. 1996).

Of particular interest is the question of whether macrophages release substances that alter calcium signals in chromaffin cells, especially by modulating voltage-gated calcium channels ($I_{Ca}$) that are the main calcium influx pathway triggering secretion (Boarder et al. 1987). Immune-system signals modulate $I_{Ca}$ in other types of cells; $I_{Ca}$ is enhanced by interleukin-1$\beta$ in vascular smooth muscle cells (Wilkinson et al. 1996). In contrast, interleukin 1$\beta$ (Plata-Salaman and ffrench-Mullen 1992) and thromboxane A$_2$ agonists (Hsu et al. 1996) both inhibit $I_{Ca}$ in hippocampal neurons, whereas prostaglandin E$_2$ (PGE$_2$) inhibits $I_{Ca}$ in sympathetic ganglion neurons (Ikeda 1992).

PGE$_2$, one of the main metabolites released by activated macrophages, has been shown to specifically bind to adrenal chromaffin cells, release intracellular calcium stores, and stimulate calcium influx through voltage-independent channels and modulate catecholamine release (Ito et al. 1991; Karaplis et al. 1989; Marley et al. 1988; Mochizuki-Oda et al. 1991; Yokohama et al. 1988). Prostaglandins are produced by cyclooxygenase (COX), which catalyzes the first two steps in their synthesis from arachidonic acid (Vane et al. 1998). Two different isoforms of the enzyme have been identified: COX-1, which is constitutively active, and COX-2, which is inducible. Typically, substantially elevated PGE$_2$ levels are only observed several hours after activation of macrophages due to induction of COX-2 activity (Lee et al. 1992; O’Sullivan et al. 1992; Pueringer and Hunninghake 1992). However, rapid production of PGE$_2$ through the constitutively active COX-1 pathway is possible as demonstrated by application of exogenous arachidonic acid to cultured macrophage cell lines (Stenson et al. 1981).

These observations raised the possibility that macrophages could inhibit $I_{Ca}$ in adrenal chromaffin cells and thereby modulate catecholamine release. Moreover, this signaling could occur on both a slow and more rapid time scale: the slow
pathway mediated by the well-documented induction of synthetic enzymes (such as COX-2) in the macrophages during periods of immune-system activation and the rapid pathway by production of PGE$_2$ or other arachidonic acid metabolites by the constitutively active COX-1, which may play a role under both pathophysiological conditions and normal physiological functioning of the gland. To address the latter possibility, we chose to investigate potential signaling between macrophages and chromaffin cells in two ways. First, as PGE$_2$ is an attractive candidate for this kind of signaling, we investigated the effects of exogenously applied PGE$_2$ on $I_{Ca}$ in chromaffin cells. The second approach involved activating a macrophage cell line with lipopolysaccharide (endotoxin) to determine whether these cells rapidly secrete signaling molecules that could alter $I_{Ca}$ activity. Our results demonstrate inhibition of $I_{Ca}$ in adrenal chromaffin cells by PGE$_2$ and an unidentified signaling molecule(s) that is released rapidly from macrophages.

**METHODS**

**Cell culture**

Chromaffin cells were prepared by digestion of bovine adrenal glands with collagenase and purified by density gradient centrifugation as previously described (Artalejo et al. 1992). The cells were plated on collagen-coated glass coverslips in 35-mm tissue culture dishes (2 ml of cell suspension; 0.15–0.2 × 10$^6$ cells/ml) and maintained in an incubator at 37°C in an atmosphere of 93% air and 7% CO$_2$ with a relative humidity of 90%. Fibroblasts were suppressed effectively with cytosine-arabinoside (10 μM), leaving relatively pure chromaffin cell cultures. Although mixed, the cultures were somewhat enriched for epinephrine containing over norepinephrine-containing cells. Half of the incubation medium was exchanged every day. This medium consisted of DMEM/F12 (1:1) (Gibco) supplemented with fetal bovine serum (10%), glutamine (2 mM), penicillin/streptomycin (100 unit/ml and 100 μg/ml), cytosine arabinoside (10 μM), and 5-fluorodeoxyuridine (10 μM).

J774.1 cells, a murine macrophage cell line (Snyderman at al. 1977), were grown in tissue culture flasks or dishes. Cells were maintained at 37°C in humidified air containing 5% CO$_2$ in RPMI culture medium (Gibco) supplemented with 5% fetal bovine serum and penicillin/streptomycin (100 unit/ml and 100 μg/ml). Cells were passaged approximately once a week by mechanical trituration.

**Electrophysiology**

Chromaffin cells were voltage clamped in the whole cell configuration of the patch-clamp technique (Hamill et al. 1981) using an Axopatch 1C amplifier (Axon Instruments) at a holding potential of −80 mV, and $I_{Ca}$ were activated by step depolarizations. Leak currents were generated by averaging 16 hyperpolarizing sweeps (steps or ramps). All the data reported in this paper were capacitance and leak subtracted. The data were filtered at 2 kHz and then digitized at 100 μs/point. Series resistance was compensated partially (~80%) using the series resistance compensation circuit of the Axopatch 1C amplifier. Electodes were pulled from microhematocrit capillary tubes (Drummond) and coated with silicone elastomer (Sylgard; Dow Corning). After fire polishing, final electrode resistances when filled with the CsCl-based patch pipette solution (see following section) were ~1.5–3.0 MΩ. Voltage protocols and data analysis were carried out in AxoBasic. Data are reported as means ± SE, and statistical significance was determined using paired or independent Student’s t-test. All recording was performed at room temperature (~23°C).

**Solutions**

Electrodes were filled with (in mM) 110 CsCl, 4 MgCl$_2$, 20 HEPES, 10 EGTA, 0.35 GTP, 4 ATP, and 14 creatine phosphate, pH = 7.3 (adjusted by CsOH) and osmolality was ~310 mOsm. The NaCl-based extracellular recording medium contained (in mM) 140 NaCl, 2 KCl, 10 glucose, 10 HEPES, and 10 CaCl$_2$ as well as 0.3–1.0 μM tetrodotoxin (TTX), pH = 7.3 (adjusted with NaOH), and the osmolality was ~310 mOsm. In a few experiments, the TTX was omitted. Nisoldipine was prepared as a stock solution (10 mM) in ethanol and stored, protected from light at −20°C. It was added to all extracellular solutions (1 μM) to block any facilitation $I_{Ca}$ (L-type) present.

PGE$_2$ (Calbiochem) was prepared as a stock solution in DMSO and aliquots frozen. Final dilutions yielded DMSO concentrations of <0.03%, which had no effect on the currents by itself. Interleukin-1β and interleukin-6 (Sigma) were prepared as stocks of 10 μg/ml and aliquots frozen until use. Ibuprofen and nordihydroguaiaretic acid (NDGA; Sigma) were both prepared fresh by dilution in H$_2$O with the addition of NaOH. Final dilution yielded no alteration on the pH of the recording or incubation medium. Lipopolysaccharide (LPS) from *Escherichia coli* (serotype 055:B5; Sigma) was dissolved in sterile H$_2$O (25 mg/ml) and aliquots stored at 4°C for 2–4 wk.

**Application of solutions and preparation of conditioned medium**

The recording bath was <1 cm in diameter with a volume of around 250–350 μl. The bath solution was gravity fed from reservoirs at a flow rate of 3–4 ml per minute, that ensured efficient perfusion of the recording chamber. Agonists and antagonists were applied to the cells by including them in the recording solution and washing them into the bath. There was a latency between switching solutions at the reservoirs and the drugs reaching the cell due to "dead space" in the tubing leading to the bath. This accounted for most of the delay seen at the reservoirs and the drugs reaching the cell due to "dead space" in the tubing to the cell. This accounted for most of the delay seen between agonist application and inhibition of $I_{Ca}$. ω-conotoxin GVIA (35–50 μl) was added directly to the bath, with the flow of extracellular solution stopped, at 10 times the desired final concentration. Thus ω-conotoxin GVIA was added at 10 μM to give a final concentration of ~1 μM.

For experiments in which J774 cells were present in the recording chamber along with the chromaffin cells, the J774 cells were grown on tissue culture dishes until almost confluent. Cells then were removed from the dish by gentle trituration and resuspended at a density of roughly 0.2–1 × 10$^6$ in NaCl-based recording medium. Once a chromaffin cell had been voltage clamped, the flow of solution through the recording chamber was stopped, and 50–75 μl of the cell suspension was added directly to the bath. The J774 cells quickly settled and adhered to the coverslip. After several minutes the flow of solution through the bath was resumed to wash away any cells that had not stuck to the coverslip.

For experiments with conditioned media the J774 cells were grown on 10 cm diameter tissue culture dishes until confluent. Cells then were washed several times with NaCl-based recording medium and then incubated for 5–10 min with 7–8 ml of either NaCl-based recording medium (control conditioned media) or NaCl-based recording medium containing 100–250 μg/ml LPS (LPS-conditioned media). The conditioned media was collected and applied directly to chromaffin cells by washing through the bath using the gravity fed perfusion system. TTX and nisoldipine were omitted from the NaCl recording medium before conditioning and were added after the solution was removed from the J774 cells.
PGE$_2$ inhibits $I_{Ca}$ in chromaffin cells in a voltage-dependent manner

PGE$_2$, an important metabolite produced by activated macrophages, was applied to the chromaffin cells by continuous perfusion through the recording chamber. In virtually every cell tested, PGE$_2$ produced a rapid, reversible inhibition of $I_{Ca}$ similar to the response illustrated in Fig. 1. Figure 1A plots peak-current amplitude as a function of time. This cell was depolarized every 10 s to $+20$ mV from $HP = -80$ mV. Approximately 70% of the current was inhibited when PGE$_2$ (300 nM) was applied. Figure 1B shows representative currents obtained during this experiment; the currents were inhibited and activation was slowed. In other experiments, PGE$_2$ was applied at concentrations of 1 nM to 1 $\mu$M, and the resulting dose-response curve (not shown) yielded an EC$_{50}$ for the inhibition of $I_{Ca}$ of $-10$ nM. Application of a supramaximal dose of PGE$_2$ (300 nM), similar to that shown in Fig. 1, produced a mean inhibition of 53 $\pm$ 4.5% ($n = 20$). Multiple applications of PGE$_2$ to the same cell produced repeated inhibition of $I_{Ca}$, suggesting there was little desensitization of the response; however, the washout was often slow and incomplete.

Activation of G-protein-coupled receptors by various transmitters/hormones can inhibit $I_{Ca}$ by multiple pathways (Hille 1994) one of which is thought to involve direct binding of the G-protein $\beta\gamma$ subunits to the channel (Herlitze et al. 1996; Ikeda 1996; for review, see Dolphin 1998). This type of inhibition is voltage dependent and characterized by slowed activation kinetics of $I_{Ca}$ similar to that shown in Fig. 1B, and relief of the inhibition by a conditioning prepulse (Bean 1989; Elmslie et al. 1990; Penington et al. 1991). Figure 2A shows an experiment where a conditioning prepulse (50-ms duration to $+100$ mV, applied 10 ms before the activation of $I_{Ca}$) relieved $65\%$ of the inhibition produced by PGE$_2$. On average these prepulses relieved $59 \pm 3\%$ ($n = 13$) of the inhibition and reversed the kinetic slowing (Fig. 2B). Please note that 1 $\mu$M nisoldipine was present throughout these experiments, so the prepulse increases in $I_{Ca}$ were due to relief of inhibition not recruitment of L-type channels.

PGE$_2$ inhibition is mediated by both PTX-sensitive and -insensitive G proteins

Most examples of voltage-dependent inhibition of $I_{Ca}$ are mediated by the Gi/Go family of G proteins and can be blocked by pertussis toxin (PTX), which disrupts the coupling of these G proteins with their receptors. We therefore tested the PTX sensitivity of the PGE$_2$ inhibition. Cells were preincubated for 18–24 h with 300 ng/ml PTX and then PGE$_2$ applied as before. In control cells (cells from the same cultures and recorded from on the same days as the treated cells), 300 nM PGE$_2$ inhibited $I_{Ca}$ by 47 $\pm$ 3.3% ($n = 11$), whereas in PTX-treated cells the
inhibited by PGE2 and that the N-type current was inhibited to suggest that both N- and P/Q-type current components were inhibited by activation of P2Y purinergic receptors. Furthermore both these current components are inhibited by nisoldipine, we have shown previously that I_{Ca} sensitive and -insensitive G proteins. Average inhibition of 300 nM PGE2 is shown for 11 control cells and 13 cells pretreated for 18–24 h with 300 ng/ml PTX. Control cells were from the same cultures and were recorded from on the same days as PTX-treated cells. Inhibition produced in PTX-treated cells was significantly reduced compared with control cells (P < 1 × 10^{-6}).

Both N- and P/Q-type I_{Ca} were inhibited by PGE2

Under our recording conditions (with L-type channels blocked by nisoldipine), we have shown previously that I_{Ca} consists of ~50% N-type and ~50% P/Q-type channels (Currie and Fox 1996, 1997). Furthermore both these current components are inhibited by activation of P2Y purinergic receptors. Consistent with these data, application of 1–2 μM α-conotoxin GVIA to selectively block the N-type I_{Ca} reduced the current amplitude by 49 ± 3.1% (n = 4), PGE2 (300 nM) inhibited the remaining P/Q-type current by 33 ± 3.7% (n = 4). Our results suggest that both PTX-sensitive and -insensitive G proteins are involved in the PGE2 response.

J774 macrophages rapidly release an inhibitor of I_{Ca} when stimulated with lipopolysaccharide (endotoxin)

From the preceding results it was clear that PGE2 inhibited I_{Ca} in adrenal chromaffin cells. Typically there is a delay of several hours after macrophage activation (due to induction of COX-2 activity) before there is a substantial elevation of PGE2 production and release. Macrophages also express the constitutively active form of the enzyme (COX-1) and so have the ability to rapidly produce PGE2 from arachidonic acid. To determine whether macrophages could rapidly produce PGE2 or other paracrine modulators of I_{Ca} on exposure to endotoxin (a lipopolysaccharide component of bacterial cell walls commonly used to activate macrophages), a mouse macrophage cell line (J774.1) was exposed to short applications (1–10 min) of high concentrations (100–250 μg/ml) of lipopolysaccharide (LPS) from Escherichia coli; chemical signals secreted by the macrophages were tested on chromaffin cell I_{Ca}.

Two approaches were used: addition of J774 cells directly to the recording chamber containing chromaffin cells followed by exposure to LPS and generation of conditioned medium from culture dishes of J774 cells and application of this directly to the chromaffin cells. The first approach is illustrated in Fig. 4. After establishing whole cell recording from a chromaffin cell, the flow of solution through the recording chamber was stopped and a suspension of J774 cells added to the chamber. The cells settled with minutes and flow of the bath was resumed. LPS was then applied to the cells, and the flow of solution through the bath was again stopped to facilitate the accumulation of any substances released from the macrophages. This produced a very rapid inhibition of I_{Ca} amplitude and a slowing of the activation kinetics (Fig. 4B). The inhibition was washed out rapidly once flow of solution through the bath was resumed. In 31 cells, the mean inhibition produced in the manner just described was 37 ± 1.2%. Application of LPS to the chromaffin cells in the absence of J774 cells had no effect on I_{Ca} (Fig. 5). Similarly when J774 cells were applied to the bath as described in the preceding text and the flow of solution stopped, there was no inhibition of I_{Ca} unless LPS was also present (Fig. 5). Prior incubation of the chromaffin cells with PTX reduced the inhibition produced by the same protocol to 12 ± 5% (n = 5).

Application of conditioned medium collected from J774 cells to chromaffin cells produced similar results (Fig. 6). J774 cells were incubated for 5–10 min with either NaCl-based recording medium to produce control conditioned medium (control), in the presence of LPS with the flow of solution through the bath stopped (LPS), and after washout of LPS with the bath solution continuously flowing (wash).

![FIG. 3. PGE2 inhibition of I_{Ca} is mediated by both pertussis-toxin (PTX)-sensitive and -insensitive G proteins. Average inhibition of I_{Ca} produced by 300 nM PGE2 is shown for 11 control cells and 13 cells pretreated for 18–24 h with 300 ng/ml PTX. Control cells were from the same cultures and were recorded from on the same days as PTX-treated cells. Inhibition produced in PTX-treated cells was significantly reduced compared with control cells (P < 1 × 10^{-6}).](http://jn.physiology.org/)

![FIG. 4. Exposure of the J774 macrophage cell line to lipopolysaccharide rapidly inhibits I_{Ca} recorded from nearby chromaffin cells. A: J774 cells were added to the recording chamber and settled onto the coverslip adjacent to the chromaffin cells within minutes. Lipopolysaccharide (LPS; 100–250 μg/ml) was washed rapidly into the chamber and the flow of solution stopped (indicated by bar) to allow accumulation of any chemical signals released from the J774 cells. Graph plots peak current amplitude vs. time and shows that LPS stimulation led to a rapid inhibition of I_{Ca}. This inhibition was rapidly washed out when fresh solution was perfused through the chamber. B: current records from the cell shown in A. Currents were recorded before application of LPS (control), in the presence of LPS with the flow of solution through the bath stopped (LPS), and after washout of LPS with the bath solution continuously flowing (wash).](http://jn.physiology.org/)
medium or NaCl-based recording medium containing LPS (100–250 μg/ml) to produce LPS-conditioned medium. This conditioned medium then was collected and applied directly to the chromaffin cells by continuous perfusion through the recording chamber. Control conditioned medium had little or no effect on \( I_{Ca} \), but LPS-conditioned medium reversibly inhibited \( I_{Ca} \) (Fig. 6, A and B). The inhibition slowed the activation kinetics of \( I_{Ca} \) and was relieved by a depolarizing prepulse (Fig. 6B). Normalizing the data to the control conditioned media showed that LPS-conditioned media inhibited \( I_{Ca} \) by 18 ± 2.3% (\( n = 12; P < 0.001 \)) and a prepulse to +100 mV reduced this inhibition to 3 ± 2% (\( n = 8; \) Fig. 7). These data are consistent with the idea that a chemical signal was rapidly (within seconds to minutes) released from the J774 cells and acted on a G-protein-coupled receptor to inhibit \( I_{Ca} \) in the chromaffin cells.

Please note that the percentage of cells responding was variable from week to week for both the conditioned media experiments and the experiments in which the J774 cells were present in the recording chamber. The reasons for this are not clear but are considered in the discussion.

**Rapidly released inhibitor is not PGE\(_2\)**

To determine if the transmitter released by the J774 cells was PGE\(_2\), the experiments shown in Figs. 6 and 7 were repeated with conditioned medium from J774 cells that had been pretreated for 1–2 h with 30–100 μM ibuprofen, which blocks activity of both COX-1 and COX-2. Control dishes of J774 cells were treated in the same manner except with the omission of ibuprofen. Ibuprofen also was present during exposure to LPS and so was present in all conditioned medium applied to the chromaffin cells. Ibuprofen itself had no direct action on \( I_{Ca} \). The amplitude of \( I_{Ca} \) was 1,403 ± 268 pA before application and 1,359 ± 264 pA (\( n = 5 \)) during application of control conditioned medium (no LPS) containing 30 μM ibuprofen.

Chromaffin cells first were exposed to LPS-conditioned medium from untreated J774 cells. After obtaining a response the cell then was washed and LPS-conditioned medium from ibuprofen-treated J774 cells was applied (Fig. 8A). There was no difference in the inhibition of \( I_{Ca} \) produced by conditioned media from control cells (17 ± 4%, \( n = 4 \)) or ibuprofen-treated cells (18 ± 3%, \( n = 4; \) Fig. 8B).

Arachidonic acid also can be metabolized by lipooxygenase enzymes to produce signaling molecules such as leukotrienes or other active metabolites. The activity of both 12-lipoxygenase and 5-lipoxygenase can be blocked by nordihydroguaiaretic acid (NDGA). To determine if a product of this pathway was responsible for the rapidly produced inhibition, experiments were performed as described in the preceding text except J774 cells were incubated for ~2 h with 30 μM NDGA. There was no difference in the mean inhibition of \( I_{Ca} \) produced by conditioned medium from control (17.5 ± 2.1%; \( n = 4 \)) or NDGA-treated (19 ± 1.9%; \( n = 4 \)) J774 cells (Fig. 8, C and D), suggesting that these pathways are not involved in the response.

It seemed unlikely that cytokines were responsible for the rapid signaling observed because it takes hours rather than
minutes after activation of macrophages before there is an up-regulation of cytokine synthesis (Lin et al. 1994; Yoo et al. 1995). However, interleukins (including IL-1 and IL-6) have been detected in the adrenal gland (Nussdorfer and Mazzocchi 1998), and IL-1β is known to inhibit \( I_{\text{Ca}} \) in hippocampal neurons (Plata-Salaman and ffrench-Mullen 1992). Therefore IL-1β and IL-6 (30–100 ng/ml) were tested to determine whether they produced an inhibition of \( I_{\text{Ca}} \) in the chromaffin cells. Chromaffin cells were exposed to the interleukins by continuous perfusion through the recording chamber. Neither interleukin had an effect on \( I_{\text{Ca}} \). In four cells tested, the amplitude of \( I_{\text{Ca}} \) was 1,126 ± 119 pA under control conditions and 1,101 ± 101 pA in the presence of IL-1β. Similarly, in five different cells, the control \( I_{\text{Ca}} \) amplitude was 995 ± 32 pA and in the presence of IL-6 was 984 ± 33 pA.

**DISCUSSION**

It is known that resident macrophages within the adrenal gland lie next to chromaffin cells. Macrophages are secretory cells that synthesize over 100 distinct products (Nathan 1987). The aim of this study was to investigate possible paracrine signaling between macrophages and adrenal chromaffin cells. In particular, these studies were focused on modulation of \( I_{\text{Ca}} \) as these channels are important targets for regulation of catecholamine release. PGE\(_2\), synthesized from arachidonic acid by the cyclooxygenase pathway, is one of the primary products secreted by macrophages. PGE\(_2\) is known to have actions on adrenal chromaffin cells including elevation of intracellular calcium and modulation of catecholamine release (Ito et al. 1991; Karaplis et al. 1989; Mochizuki-Oda et al. 1991; Yokohama et al. 1988) but its actions on \( I_{\text{Ca}} \) were unknown. However, PGE\(_2\) does inhibit \( I_{\text{Ca}} \) in sympathetic ganglion neurons (Ikeda 1992), which are ontogenetically similar to chromaffin cells. It therefore seemed to be an attractive candidate to participate in paracrine inhibition of \( I_{\text{Ca}} \) in chromaffin cells.

Our results demonstrate that PGE\(_2\) produced a robust inhibition of both N- and P/Q-type \( I_{\text{Ca}} \) in virtually every cell tested. In addition to the reduction in current amplitude, the activation kinetics of \( I_{\text{Ca}} \) were slowed. Conditioning prepulses reversed the slowing of activation and partially relieved the reduction in current amplitude. These features are characteristic of G-protein-mediated inhibition of N- and P/Q-type \( I_{\text{Ca}} \) in chromaffin cells, neurons, and other cell types (Bean 1989; Currie and Fox 1996; Dolphin 1995; Elmslie et al. 1990; Hille 1994; Penington et al. 1991). Most examples of this type of inhibition are mediated by PTX-sensitive G proteins. In the chromaffin cells,
it appears that PGE₂ couples to both PTX-sensitive and -insensitive G proteins to inhibit I_{Ca} similar to results obtained in sympathetic neurons (Ikeda 1992). It is not clear whether the same PGE₂ receptor couples to multiple G proteins or whether there are multiple receptors activated concomitantly. There are very few readily available, selective pharmacological agents for prostanooid receptors so the subtype(s) involved in this response were not characterized.

Macrophages express the constitutive (COX-1) as well as the inducible (COX-2) form of cyclooxygenase. Substantially elevated production of PGE₂ is usually not seen until several hours after activation of macrophages in part due to induction of COX-2 (Lee et al. 1992; O’Sullivan et al. 1992; Pueringer and Hunninghake 1992). However, it is possible for PGE₂ to be produced rapidly by COX-1 as demonstrated by addition of exogenous arachidonic acid to macrophage cell lines (Stenson et al. 1981). This raised the possibility that macrophages could signal rapidly to chromaffin cells through the production of PGE₂ (or other arachidonic acid metabolite) in addition to slower signaling through induction of synthetic enzymes such as COX-2 or synthesis of cytokines and related products.

This rapid signaling pathway was investigated using a mouse macrophage cell line (J774.1) stimulated with high concentrations of lipopolysaccharide (LPS), commonly used to activate macrophages. Addition of J774 cells to a recording chamber containing chromaffin cells had no effect on I_{Ca} unless LPS also was added to the bath. With LPS and J774 cells present, and the flow of solution through the bath stopped to allow accumulation of any released chemical signals, I_{Ca} amplitude was inhibited rapidly and activation kinetics were slowed; prepulses relieved a portion of the inhibition. PTX pretreatment of chromaffin cells significantly reduced the inhibition. Thus these results suggest the existence of rapid signaling between the immune system and chromaffin cells.

To confirm these observations, conditioned medium was collected from J774 cells and applied directly to chromaffin cells. This produced a comparable inhibition of I_{Ca}. Interestingly, responses both to LPS-activated macrophages present alongside chromaffin cells and to conditioned media were much less consistent than direct application of PGE₂. The proportion of cells responding to conditioned medium was low and variable from week to week. The reasons for this variability are uncertain but may include: only a subpopulation of the chromaffin cells respond to the macrophage-derived chemical mediator; variability in J774 cell density and/or properties; variability between batches of LPS; and variable amounts of the macrophage-derived mediator produced and/or potential degradation or decay of this chemical once released. Nonetheless despite the problems arising from this variability, responses from many cells were obtained allowing the inhibition to be characterized.

The rapid inhibition produced by the LPS-conditioned medium was not mediated by PGE₂ as blocking COX activity in the J774 cells using ibuprofen did not suppress the inhibition. The lipooxygenase pathway, which metabolizes arachidonic acid into leukotrienes, did not appear to be involved in the rapid signaling, as blockade of this pathway did not prevent inhibition of I_{Ca} by the LPS-conditioned medium. It is possible that there are multiple signaling molecules involved in the response and at present the identity of the inhibitor(s) remains unknown. The observation that the macrophage-derived inhibitor was stable in LPS-conditioned medium suggests it is not nitric oxide, which has a short half life once released from macrophages. The same is true for the eicosanoids, which are short-lived arachidonic acid metabolites produced by the cytochrome P450 pathway (Imig 1999). Activation of macrophages stimulates the synthesis of cytokines so it appears unlikely that large amounts could be released rapidly enough to account for the inhibition reported in this paper. However, IL-1 and IL-6 have been reported in the adrenal gland (Nussdorfer and Mazzocchi 1998) and IL-1β inhibits I_{Ca} in hippocampal neurons (Plata-Salaman and ffrench-Mullen 1992). Both IL-1β and IL-6 were applied directly to chromaffin cells but neither was found to have an effect on I_{Ca}.

Paracrine signaling within the adrenal gland is likely to be a complex web of interactions. Chromaffin cells are known to influence the functioning of adrenocortical cells; these cells send signals back to the chromaffin cells as well (Nussdorfer 1996; Pignatelli et al. 1998). It is also becoming apparent that resident macrophages can participate in paracrine signaling within the adrenal gland; locally produced cytokines act on the adrenal cortex to stimulate steroid production (Marx et al. 1998; Nussdorfer and Mazzocchi 1998). Conversely, glucocorticoids are known to suppress cytokine production and induction of COX-2 (Buckingham et al. 1996; Turnbull and Rivier 1995), which contributes to the negative feedback immunosuppressive actions of the hypothalamic-pituitary-adrenal axis. There are fewer reports of paracrine interactions between macrophages and chromaffin cells, but these also may operate bidirectionally; catecholamines are known regulators of immune-system function (Coffey and Hadden 1985; Johnson et al. 1981) and histogranin, a recently described peptide released from chromaffin cells, has been shown to stimulate immune cells including macrophages (Lemaire et al. 1995). Conversely, stimulation of catecholamine secretion by a peptide released from monocytes (Jones et al. 1993; Roberts et al. 1996) and the induction of c-fos immunoreactivity in chromaffin cells by NT4, which is contained in a subpopulation of the resident macrophage cells (Schober et al. 1998) have been reported. PGE₂ also has been reported to augment or directly stimulate catecholamine release possibly by stimulating extracellular calcium influx through voltage-independent channels (Karaplis et al. 1989; Marley et al. 1988; Yokohama et al. 1988). However, concentrations of PGE₂ in the nanomolar range suppress catecholamine release stimulated by nicotine (Karaplis et al. 1989). The results reported in this paper suggest that PGE₂ may suppress catecholamine release by reducing calcium influx through I_{Ca}. Further study will be required to elucidate the precise balance between these two seemingly opposing mechanisms on catecholamine release.

In summary, this paper identifies a novel paracrine signaling pathway between macrophages and adrenal chromaffin cells that may regulate catecholamine release through modulation of I_{Ca}. It is possible that during periods of immune-system activation (infection), induction of COX-2 may elevate locally produced PGE₂, suppress calcium influx into the chromaffin cells, and oppose the increased sympathetic stimulation to help prevent excessive elevations in circulating catecholamines. The rapid production of a paracrine inhibitor(s) of I_{Ca} also lends a further dimension to macrophage-chromaffin cell interactions. It may facilitate a more dynamic signaling pathway that potentially could play a role in the normal physiological
functioning of the adrenal medulla as well as during periods of immune-system activation.

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REFERENCES


CURRIE, K.P.M. and FOX, A. P. ATP serves as a negative feedback inhibitor of voltage-gated Ca\textsuperscript{2+} channel currents in cultured bovine adrenal chromaffin cells. Neuron 16: 1027–1036, 1996.


