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Serum-induced membrane depolarization in quiescent fibroblasts: activation of a chloride conductance through the G protein-coupled LPA receptor

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Serum stimulation of quiescent fibroblasts leads to a dramatic depolarization of the plasma membrane; however, the identity of the active serum factor(s) and the underlying mechanism are unknown. We find that this serum activity is attributable to albumin-bound lysophosphatidic acid (LPA) acting on its own G protein-coupled receptor, and that membrane depolarization is due to activation of an anion conductance mediating Cl^- efflux. This depolarizing Cl^- current can also be activated by thrombin and neuropeptide receptors; it is distinct from volume-regulated Cl^- currents. Activation of the Cl^- current consistently follows stimulation of phospholipase C and coincides with remodelling of the actin cytoskeleton, which is regulated by the Ras-related GTPase Rho. However, the response is not due to Ca^{2+}/protein kinase C signalling and requires neither Rho nor Ras activation. The results indicate that in quiescent fibroblasts, LPA and other G protein-coupled receptor agonists evoke membrane depolarization by activating a new type of Cl^- channel through a signalling pathway that is closely associated with phosphoinositide hydrolysis, yet independent of known second messengers.

Keywords: chloride current/fibroblasts/G protein-coupled receptor/lysophosphatidic acid/membrane depolarization

Introduction

Among the earliest responses of quiescent cells to mitogenic stimulation is a variety of ionic events. These include: an immediate rise in cytosolic [Ca^{2+}]; an increase in intracellular pH due to enhanced Na^+/H^+ exchange; and, in serum-stimulated cells, a dramatic long-lasting depolarization of the plasma membrane. While much has been learned about Ca^{2+} signalling and activation of Na^+/H^+ exchange by growth factors, very little is still known about the acute loss of membrane potential following serum stimulation of quiescent cells.

It is many years since the mitogenic fraction of serum was found to rapidly depolarize the plasma membrane of contact-inhibited fibroblasts by as much as 50 mV (Hülser and Frank, 1971; Moolenaar *et al.*, 1982). Acute sustained membrane depolarization is also observed in serum-stimulated mouse neuroblastoma cells (Moolenaar *et al.*, 1981). Prolonged membrane depolarization in activated cells may serve diverse physiological functions, ranging from the control of membrane excitability (in excitable cells) and modulation of Ca^{2+} signalling to the regulation of protein translocation across the membrane (Cao *et al.*, 1995; Otero *et al.*, 1995). As yet, the identity of the depolarization-inducing serum factor(s) is not known, neither is the underlying ionic mechanism, although it has been hypothesized that depolarization is due to activation of non-selective cation channels by serum-borne polypeptides (Hülser and Frank, 1971; Moolenaar *et al.*, 1981; Frace and Gargus, 1989; Lovisolo *et al.*, 1992).

In the present study we set out to determine the nature of the active serum factor and to investigate how it induces membrane depolarization in serum-deprived fibroblasts. We show here that (one of) the responsible serum factor(s) is lysophosphatidic acid (LPA), an albumin-bound phospholipid mitogen that is released from activated platelets to influence target cells by activating a specific G protein-coupled receptor (for review see Jalink *et al.*, 1994; Moolenaar, 1995a,b). We demonstrate that LPA-induced membrane depolarization in quiescent fibroblasts is due to activation of a new type of Cl^- channel (with $Cl^->I^-$ selectivity), rather than a non-selective cation channel as believed previously. Furthermore, we show that LPA action can be mimicked by other G protein-coupled receptor agonists, notably thrombin, endothelin and neurokinin A acting on its heterologously expressed receptor.

Recent studies have shown that, in fibroblasts, the LPA receptor couples to G protein-mediated stimulation of phospholipase C (PLC) with consequent Ca^{2+} mobilization, inhibition of adenylyl cyclase, and activation of the small GTP-binding proteins Ras and Rho (Jalink *et al.*, 1994; Moolenaar, 1995a,b). LPA-induced Ras-GTP accumulation accounts for the eventual stimulation of DNA synthesis, while active Rho mediates rapid formation of focal adhesions and actin stress fibres. We have examined to what extent any of these signalling cascades triggered by LPA accounts for activation of the newly found Cl^- conductance. Our results indicate that activation of the Cl^- conductance correlates strikingly with stimulation of phosphoinositide-specific PLC and Rho-mediated cytoskeletal remodelling, yet cannot be explained by known signalling pathways.

Results

Effects of serum on the membrane potential of Rat-1 cells

The resting membrane potential of quiescent, serum-deprived Rat-1 cells in monolayer is –66.9 ± 0.5 mV

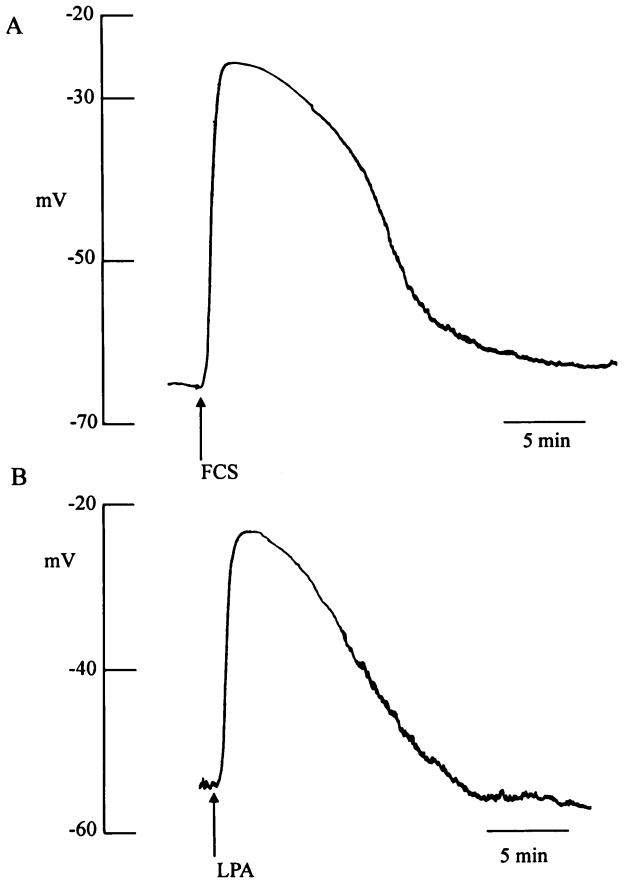

Fig. 1. (**A** and **B**) Membrane depolarization induced by serum (FCS; 10% v/v) and 1-oleoyl-LPA (1 μM) in confluent, serum-starved Rat-1 cells. Membrane potentials were recorded under current–clamp using the whole cell perforated patch–clamp technique.

(mean ± SEM; $N = 53$), as measured by either direct intracellular recording or the whole cell patch–clamp method (current–clamp mode). Previous studies have shown that the resting potential of normal fibroblasts, including Rat-1 cells, is dominated largely by the K^+ equilibrium potential, with little or no contribution of Na^+ and Cl^- ions (Moolenaar *et al.*, 1982; van der Valk *et al.*, 1987). Of note, we found that single Rat-1 cells lacking intercellular contacts have a much less negative membrane potential than confluent cells (mean ± SEM: –31.9 ± 3.7 mV; $N = 12$); the ionic basis of this phenomenon was not further investigated. The total membrane resistance of confluent Rat-1 cells (~20 MOhm) was much lower than that for single cells (~500 MOhm), consistent with monolayer cells being electrically coupled via gap junctions.

As shown in Figure 1A, addition of whole serum (5–10% v/v) to confluent Rat-1 cells results in a rapid, long-lasting membrane depolarization. The membrane potential rises by ~50 mV within seconds to a plateau value between –15 and –20 mV (mean ± SEM: –18.5 ± 5.4 mV; $N = 11$), which is maintained for a few minutes. During the next 10–30 min the membrane slowly repolarizes (in the continuous presence of serum) to the pre-stimulation potential value. In other experiments (not shown), full repolarization took as long as 40–60 min, as documented earlier for contact-inhibited rat embryo fibroblasts (Hülser and Frank, 1971) and human skin fibroblasts (Moolenaar *et al.*, 1982).

The response to serum is attributable to albumin-bound LPA

In an initial screen, we found that peptide growth factors such as EGF (50 ng/ml), PDGF (50 ng/ml), insulin (5 μg/ml), basic FGF (100 ng/ml) and TGF-β (20 ng/ml) failed to mimic the depolarizing action of whole serum on Rat-1 cells; only with PDGF we sometimes observed a relatively small, very slow depolarization (not shown). The depolarizing serum activity resisted extensive boiling, was insensitive to disulfide reduction using dithiothreitol, and could not be removed by dialysis (cut-off >10 kDa) against aquaeous buffer; however, dialysis against methanol largely removed the depolarizing activity (not shown). These biochemical characteristics are very similar to those of albumin-bound LPA, a platelet-derived phospholipid mitogen which is present in serum at biologically active concentrations (whole serum contains 2–20 μM LPA; Moolenaar, 1995b; see also Tigyi and Miledi, 1992). Indeed, when serum-borne LPA was separated from other serum lipids using 2-dim. TLC (Eichholtz *et al.*, 1993) and the LPA spot was scraped off the plate and then applied to Rat-1 cells, immediate membrane depolarization ensued. Similarly, 1-oleoyl- or 1-palmitoyl-LPA (1 μM) from commercial sources gave a response indistinguishable from that to whole serum (Figure 1B). Half-maximal responses were observed at about 10–20 nM 1-oleoyl-LPA, a value similar to that reported for other LPA-induced early events (Jalink *et al.*, 1994; Moolenaar, 1995a,b).

Other lysophospholipids, platelet-activating factor, 1-oleoyl-glycerol and phosphatidic acid were without any effect (not shown). When cells were first challenged with serum, they were completely unresponsive to subsequent addition of LPA. When the order of addition was reversed (first LPA and then serum) membrane depolarization was still detectable, in agreement with the notion that serum contains additional albumin-bound lipids (methanol-soluble) with LPA-like activity (Tigyi and Miledi, 1992; Moolenaar, 1995b); the identity of these factors remains to be determined.

The electrophysiological studies were complemented by fluorimetric potential measurements using the lipophilic anion bis-oxonol. Following membrane depolarization, negatively charged bis-oxonol enters the cells where it partitions into lipid-rich intracellular components and thereby undergoes an increase in fluorescence. Figure 2A shows a typical example of the dramatic increase in intracellular bis-oxonol fluorescence following LPA addition, as visualized by confocal microscopy. Note that every cell in the culture shows a voltage response, as one would expect for cells that are electrically coupled. Figure 2B shows the increase in bis-oxonol fluorescence in LPA-treated cells; the relatively slow response time observed in these experiments is due to the slow redistribution mechanism of bis-oxonol.

Ionic basis of LPA-induced membrane depolarization: activation of an inward Cl^- current

To determine the ionic basis of the LPA-induced membrane depolarization, we recorded whole-cell currents under

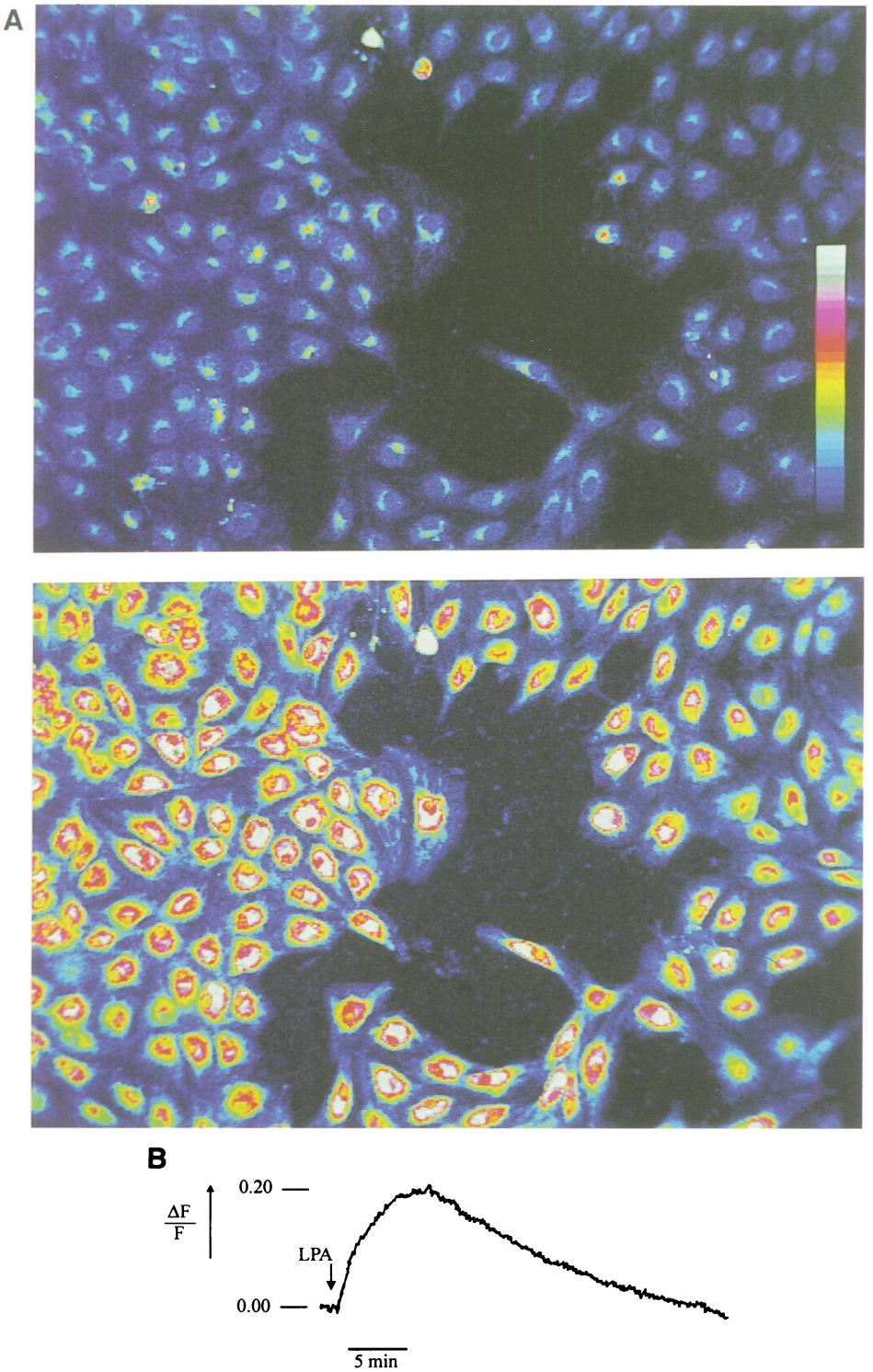

Fig. 2. (**A**) Pseudo-colour bis-oxonol images of Rat-1 cells before and after LPA addition. Cells were incubated in DMEM containing bis-oxonol (200 nM) and membrane depolarization was visualized as an increase in fluorescence due to accumulation of the negatively charged dye into lipid-rich compartments (such as endoplasmic reticulum and Golgi, but not mitochondria which have a negative membrane potential). Fluorescence was monitored by confocal microscopy and intensity displayed in pseudo-colour. Upper panel: serum-starved control cells. Lower panel: same cells exposed to 1-oleoyl-LPA (5 μM) for 5 min. (**B**) LPA-induced membrane depolarization measured as relative increase in bis-oxonol fluorescence (F). The marked difference in rise time between the fluorimetric and electrophysiological recordings is due to the relatively slow uptake and intracellular redistribution of bis-oxonol.

voltage–clamp using the perforated patch technique. At a holding potential of –60 mV, both serum [fetal calf serum (FCS); 10%] and LPA (1 μM) evoke a rapid inward current with amplitudes of up to 1000 pA (mean for FCS: 710 ± 44 pA, N = 4; mean for LPA: 770 ± 99 pA, N = 23), as measured in monolayer cells (Figure 3A and B). In single cells clamped at –60 mV, LPA and FCS failed to induce a significant inward current in a reproducible

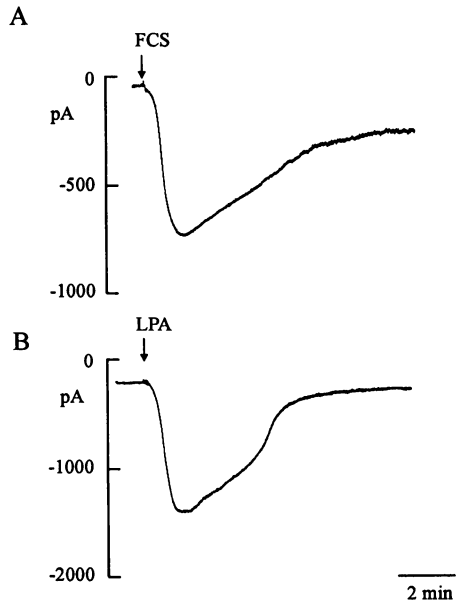

Fig. 3. (**A** and **B**) Whole cell inward currents induced by FCS (10% v/v) and 1-oleoyl-LPA (1 μM). Voltage–clamp holding potential was –60 mV.

manner. Therefore, only cells in monolayer were used for analysis, despite the fact that interpretation of voltage–clamp data from such cells is hampered by the presence of gap junctions and, hence, lack of proper space–clamp conditions; this prevents, for example, reliable determination of current reversal potentials. In addition, we observed that fibroblast gap junctions close within minutes after LPA addition, resulting in a large increase in input resistance (F.R.Postma *et al.*, manuscript in preparation); this explains why the voltage–clamp and current–clamp recordings show different time courses.

Replacement of extracellular Na^+ by either choline$^+$ or *N*-methyl-D-glucamine$^+$ (Figure 4A) left the inward current unaltered, both in amplitude and kinetics, indicating that the current is not carried by Na^+ and arguing against a role for non-selective cation channels. Furthermore, the potential value to which the cells depolarized, as measured under current–clamp, was not significantly altered after Na^+ removal (Na^+ value: –20.3 ± 2.4 mV, N = 12; choline$^+$ value: –17.0 ± 1.7 mV, N = 7). In marked contrast, when extracellular Cl^- was reduced 2-fold (by replacing Cl^- with gluconate), the amplitude of the LPA-induced current measured at –60 mV was immediately enhanced (Figure 4B); under these conditions, the cells depolarized to –1.6 ± 1.5 mV (N = 4). These results strongly suggest that the current flows through Cl^- channels, and thus represents net Cl^- efflux driven by an outward electrochemical Cl^- gradient. We therefore tested the effects of known anion channel blockers. Niflumic acid (NFA; 100 μM), a broad spectrum anion-channel blocker (White and Alwyn, 1990), inhibits the LPA-induced current (Figure 4C), whereas 1 mM DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) was ineffective.

Additional evidence that Cl^- is the responsible charge carrier comes from anion efflux measurements using $^{125}I^-$ and $^{36}Cl^-$ as tracers. Various Cl^- channels are permeable

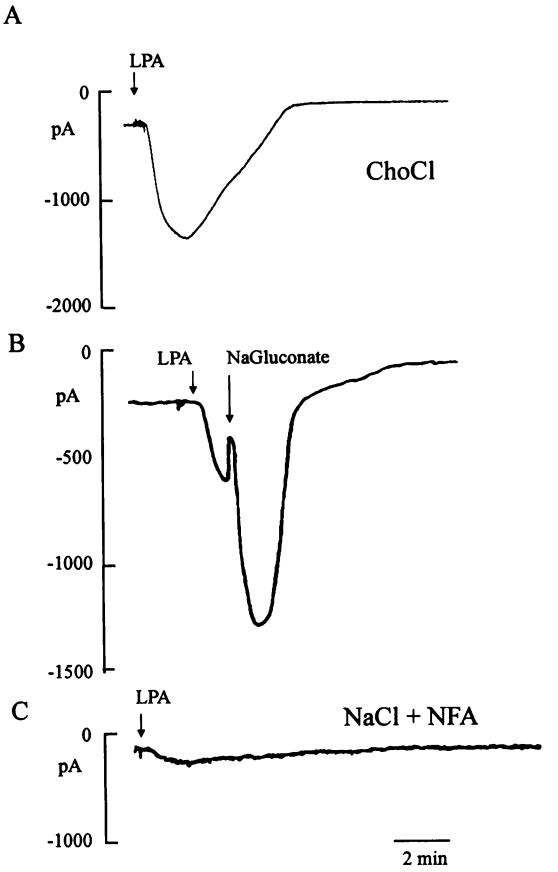

Fig. 4. LPA-induced inward currents as a function of extracellular Na^+ and Cl^- replacement and the presence of NFA. (**A**) NaCl was replaced by choline chloride. (**B**) Extracellular Cl^- was suddenly reduced (~2-fold) by addition of the required volume of Na-gluconate, as indicated. (**C**) Cells were pre-incubated with NFA (100 μM) for 5 min. For further details see text.

to I^-, but many other Cl^- transporters fail to carry I^- (Clancy *et al.*, 1990; Vaandrager *et al.*, 1991). Therefore, I^- flux measurements are a more selective assay of Cl^- channel function than Cl^- flux measurements. As shown in Figure 5, both LPA and serum rapidly stimulate unidirectional efflux of $^{125}I^-$ from $^{125}I^-$-loaded cells, with LPA acting somewhat less efficiently than serum. A similar efflux response was observed with $^{36}Cl^-$ as a tracer, but the basal efflux rate of Cl^- was significantly higher than that of I^-. From the calculated Cl^-/I^- efflux ratio (Table I) we infer that the LPA-activated Cl^- channel has a 3-fold greater permeability for Cl^- than for I^-. In parallel experiments, we found that LPA hardly affects basal $^{86}Rb^+$ efflux in Rat-1 cells (not shown); again, this finding argues against involvement of non-selective cation channels in LPA action.

Effects of endogenously and heterologously expressed G protein-coupled receptors

We next examined other agents and G protein-coupled receptor agonists for their ability to mimic LPA action in Rat-1 cells, using bis-oxonol fluorescence. Substantial membrane depolarization is also observed after addition of 10 μM AlF_4^-, which directly activates heterotrimeric but not small GTP-binding proteins (Kahn, 1991). Cells

exposed to AlF_4^- fail to respond to subsequent addition of LPA (Figure 6A), supporting the view that LPA-induced depolarization is G protein-mediated.

We found that in addition to serum and LPA, the peptide endothelin, which is a potent activator of PLC in Rat-1 cells (van der Bend *et al.*, 1992; van Corven *et al.*, 1992), the mitogenic protease thrombin (1 U/ml) as well as a thrombin receptor-activating peptide (TRP; 100 μM) all induce dramatic membrane depolarization, very similar to that observed with serum and LPA (Figure 6B and C), accompanied by enhanced Cl^- efflux (shown for endothelin in Figure 5). Activation of endogenous β-adrenergic receptors by isoproterenol (1 μM; van Corven *et al.*, 1989) was without effect. There was no cross-desensitization detectable between the responses to LPA, endothelin and thrombin (not shown), consistent with all three agonists acting on distinct receptors.

Further evidence that membrane depolarization in fibroblasts is induced by a subclass of G protein-coupled receptors comes from transfection studies with the receptor for neurokinin A (NKA). We recently showed that the human NKA receptor, when stably expressed in Rat-1 cells, couples to stimulation of PLC (Alblas *et al.*, 1995). NKA stimulation of these transfectants triggers an LPA-like membrane depolarization (Figure 6D); the responses to NKA and LPA did not show cross-desensitization. All three agonists (NKA, endothelin, TRP) induced an inward Cl^- current with similar kinetics and amplitude as observed with LPA (Figure 7).

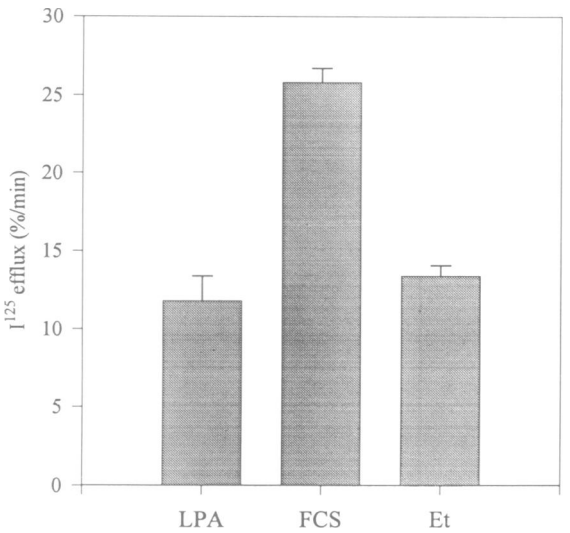

Fig. 5. Effects of LPA, whole serum and endothelin on $^{125}I^-$ efflux in Rat-1 cells. Fractional efflux was determined as described in Materials and methods. LPA (1 μM), FCS (10%) and endothelin (100 nM) were included in efflux media at 2 min after starting the efflux experiment. Data are means ± SD (N = 4).

The receptor-activated current is distinct from swelling-induced Cl^- currents

Enhanced Cl^- efflux is commonly observed after cell swelling induced by hypotonicity (regulatory volume decrease); this raises the possibility that the observed Cl^- current is secondary to agonist-induced cell swelling. Swelling induced by 30% hypotonicity evokes a small, slowly developing inward current as well as Cl^- efflux in Rat-1 cells (Figure 8A). However, the LPA-activated Cl^- efflux pathway is additive to and clearly distinct from the

Table I. Selectivity of anion efflux pathways in confluent Rat-1 cells

Stimulus	$^{36}Cl^-$ to $^{125}I^-$ efflux ratio
Control	2.13 ± 0.19
1-oleoyl-LPA (5 μM)	2.86 ± 0.22
Serum (10%)	1.32 ± 0.18
Hypotonicity (30%)	0.12 ± 0.02
Ca^{2+} ionophore (5 μM)	0.39 ± 0.07

Fractional isotope efflux from cells grown to confluence in 6-well culture plates was measured in the presence of 100 μM bumetanide to inhibit cation/Cl^- co-transport, as described in Materials and methods. Efflux ratios were calculated as described in Vaandrager *et al.* (1991). Values are expressed as means ± SEM (N = 4).

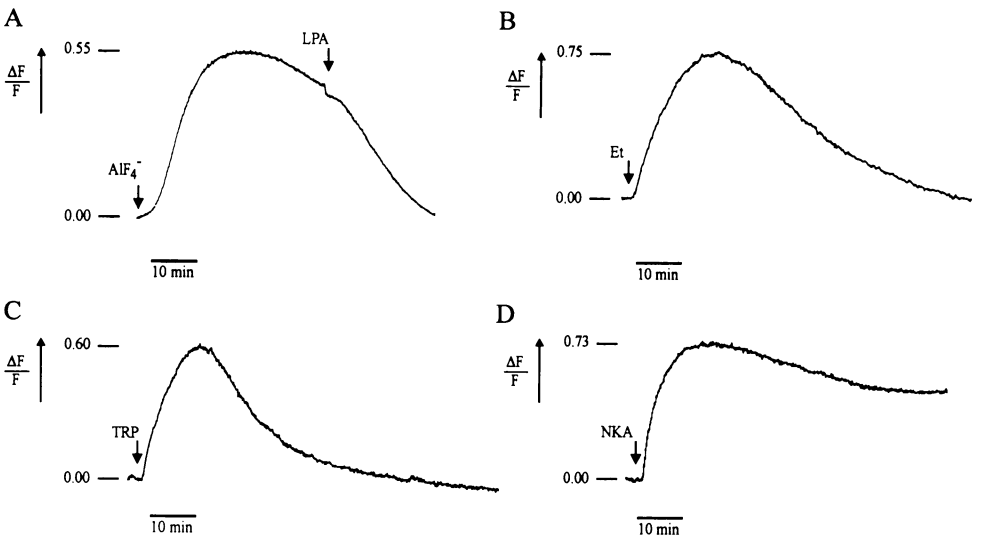

Fig. 6. LPA-like membrane depolarization in Rat-1 cells induced by AlF_4^- and G protein-coupled receptor agonists, measured by bis-oxonol fluorescence (F). (**A**) AlF_4^- was added at a final concentration of 30 μM; subsequently, LPA was added at 5 μM as indicated (arrow). (**B**) Et, endothelin (100 nM) and (**C**) TRP (100 μM). (**D**) Addition of NKA (1 μM) to Rat-1 cells stably transfected with the human NKA receptor cDNA.

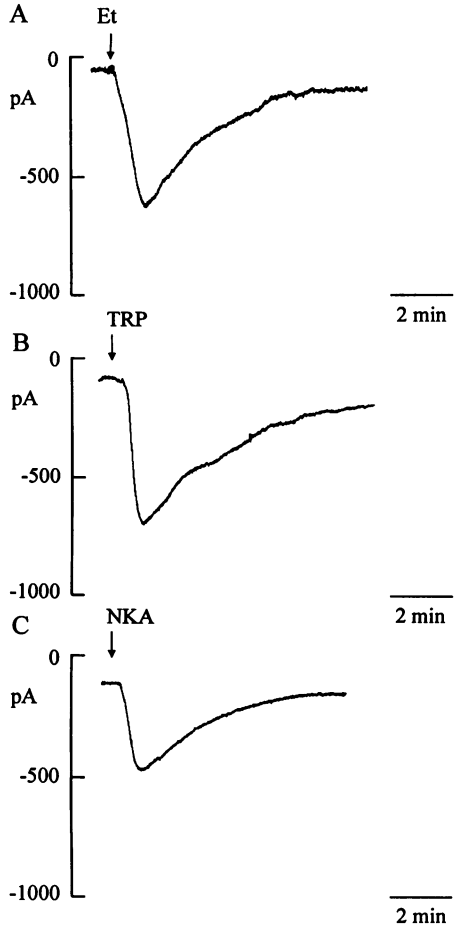

Fig. 7. Whole cell currents recorded under voltage–clamp. Et, endothelin (100 nM); TRP (100 μM); NKA (1 μM) added to Rat-1 cells expressing the human NKA receptor. Holding potential was –60 mV.

one induced by swelling, as revealed by both electrophysiological (Figure 8A) and isotope efflux experiments (Table I). While the Cl^-/I^- efflux ratio for LPA-stimulated cells is 2.9, it is as low as 0.1 for the hypertonicity-induced efflux ratio (Table I). Thus, the swelling-induced channel is considerably more permeable to I^- than to Cl^-, just opposite to what is observed for the LPA-induced efflux pathway.

Dissection of signalling pathways: association with PLC

We next examined which G protein pathway underlies activation of the depolarizing Cl^- current. In fibroblasts, the LPA receptor couples to: (i) stimulation of PLC; (ii) inhibition of adenylyl cyclase; (iii) activation of Ras and the downstream Raf-MAP kinase cascade and (iv) Rho-mediated cytoskeletal reorganization together with tyrosine phosphorylation of focal adhesion proteins (Moolenaar, 1995a,b). Of these pathways, inhibition of adenylyl cyclase and activation of Ras signalling are sensitive to pertussis toxin (PTX). Pre-treatment of the cells with PTX (100 ng/ml; 12 h) inhibited neither LPA- nor serum-induced membrane depolarization and inward currents, indicating that G_i is not involved and that neither Ras signalling nor inhibition of adenylyl cyclase is required.

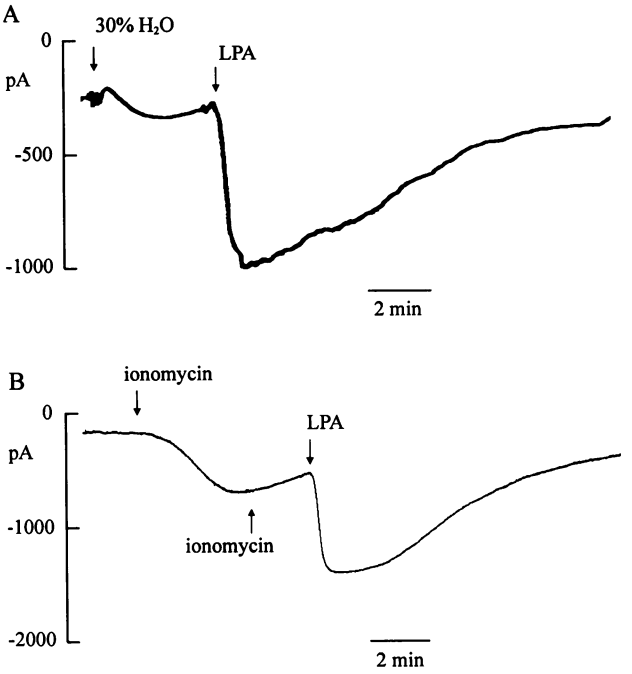

Fig. 8. LPA-induced inward current is distinct from swelling- or Ca^{2+}-regulated currents. **(A)** Cell swelling was induced by exposing confluent Rat-1 cells to hypotonic medium (30% H_2O). Thereafter, 1-oleoyl-LPA was added at 1 μM. **(B)** Cells were treated twice with ionomycin (50 nM) and then with LPA (1 μM), as indicated. Holding potential was –60 mV.

Table II. Correlation beteen PLC activation, actin reassembly and Cl^--mediated membrane depolarization in quiescent Rat-1 cells

Addition	Total inositol phosphates (fold stimulation)[a]	Stress fibres[b]	Depolarization[c]
None	1.0	–	–
FCS (10%)	4.4	++	++
1-oleoyl-LPA (5 μM)	3.2	++	++
Endothelin (100 nM)	22.5	+++	+++
TRP (100 μM)	n.d.	++	++
NKA (1 μM)[d]	2.7	+	+
EGF (50 ng/ml)	1.0	–	–

[a][^{3}H]Inositol-labelled Rat-1 cells were stimulated with agonist for 30 min and formation of total [^{3}H]inositol phosphates was measured in the presence of 10 mM LiCl as described by Alblas *et al.* (1995). SEM was <10% (N = 3). Control value was 1150 ± 39 d.p.m./well; n.d., not determined.
[b]Formation of actin stress fibres was assessed semi-quantitatively at 5 min after agonist addition, as described in Materials and methods. Control cells were virtually devoid of stress fibres. The response to agonists was defined as: +, moderate; ++, intermediate; and +++, maximal formation of new stress fibres spanning the entire cytoplasm.
[c]The degree of membrane depolarization was assessed semi-quantitatively from the amplitude and duration of the voltage response.
[d]Added to Rat-1 cells expressing the human NKA receptor (Alblas *et al.*, 1995).

We note that activation of the Cl^- conductance closely correlates with stimulation of PLC. Thus, all the receptor agonists that activate depolarizing Cl^- efflux in Rat-1 cells (LPA, thrombin, endothelin and NKA) also activate PLC with consequent Ca^{2+} mobilization (Table II); furthermore, these agonists also induce rapid actin assembly (see below). We found that a putative PLC inhibitor, the

aminosteroid U-73122 (10 μM; Thompson *et al.*, 1991), blocks LPA-induced depolarization (not shown); however, this result is difficult to interpret, as U-73122 by itself induces a depolarizing current of unknown ionic origin in Rat-1 cells (F.R.Postma, unpublished results).

We tested whether the Cl^- current is mediated by a rise in cytosolic Ca^{2+}. The Ca^{2+} ionophore ionomycin (50 nM) elicited a slowly developing inward current in all cells tested. The ionomycin-induced current was similar to the receptor-activated currents with respect to sensitivity to NFA and removal of extracellular Cl^-. However, while a second addition of ionomycin had no effect, indicative of saturation, subsequent addition of LPA immediately elicited an inward current with a much faster rise time (Figure 8B). Conversely, pre-incubation of the cells with thapsigargin (200 ng/ml), which discharges internal Ca^{2+} stores (Thastrup *et al.*, 1990; Lytton *et al.*, 1991), left the response to LPA unaltered. Furthermore, the Cl^-/I^- efflux ratio observed with Ca^{2+} ionophore differs from that seen with LPA (Table I), indicating that different efflux pathways are involved. Taken together, these results indicate that the LPA-induced Cl^- current is not mediated by a rise in cytosolic Ca^{2+}.

Our results also exclude protein kinase C (PKC) as a mediator of the Cl^- current: addition of the phorbol ester TPA (100 ng/ml) has no effect on membrane potential, while long-term pre-incubation of the cells with high doses of TPA (1 μg/ml) to down-regulate PKC did not prevent LPA-induced depolarization; furthermore, the potent PKC inhibitors staurosporine (1 μM) and Ro-31–8220 (5 μM; Davis *et al.*, 1989) did not inhibit LPA action. These results also imply that LPA-induced phospholipase D activation, which is mediated by PKC (van der Bend *et al.*, 1992), is not involved in membrane depolarization.

Although LPA is a rather poor inducer of arachidonic acid release in fibroblasts (van Corven *et al.*, 1989), we tested the effects of specific inhibitors of phospholipase A_2 (PLA_2) and arachidonate-metabolizing enzymes. AACOCF$_3$ (arachidonoyl trifluoromethyl ketone; 10 μM), a specific competitive inhibitor of cytosolic PLA_2 (Bartoli *et al.*, 1994), had no effect on the Cl^--mediated depolarization. Similarly, two different inhibitors of 5-lipoxygenase, MK-886 and A-64077 (or Zileuton; Ford-Hutchinson *et al.*, 1994; Rouzer *et al.*, 1990) and the cyclo-oxygenase inhibitor indomethacin (1 μM) were without effect in both patch–clamp and efflux experiments (not shown). These results strongly argue against a role for PLA_2 and/or arachidonate metabolites in Cl^- channel activation. Finally, the cell-permeable cyclic nucleotides 8Br-cAMP and 8Br-cGMP (1 mM) had no detectable effect on membrane potential or currents, neither did these analogues affect LPA action.

Depolarization parallels Rho-dependent cytoskeletal changes

In serum-starved fibroblasts, LPA and serum stimulate rapid formation of focal adhesion and actin stress fibres, accompanied by tyrosine phosphorylation of focal adhesion proteins in a Rho-dependent manner (Ridley and Hall, 1992; Kamugai *et al.*, 1993; Hordijk *et al.*, 1994; Seufferlein and Rozengurt, 1994). We found that, in addition to LPA and serum, all depolarizing receptor agonists (endothelin, thrombin, NKA, but not EGF) rapidly induce actin stress fibres in Rat-1 cells (Table II), with a time course that roughly parallels activation of the depolarizing Cl^- current. Pre-treatment with the *Clostridium botulinum* C3 ADP-ribosyltransferase, which inactivates Rho, blocks stress fibre formation by the above agonists, but has no effect on activation of the Cl^- conductance. Similarly, disruption of the actin cytoskeleton by cytochalasin D (5 μg/ml) left agonist-induced membrane depolarization unaltered. Finally, the tyrosine kinase inhibitors genistein (50 μM), tyrphostin 25 (150 μM; 30 min pre-treatment) and herbimycin A (4 μM; 16 h pre-treatment) had no effect on the LPA-induced inward current. These data indicate that, although Cl^--mediated depolarization closely parallels Rho-mediated cytoskeletal changes and tyrosine phosphorylation of focal adhesion proteins, functional Rho, tyrosine kinase activity and an intact actin cytoskeleton are not required for LPA to activate the Cl^- conductance.

Discussion

The present study provides new answers to the long-standing question of how serum depolarizes the plasma membrane of quiescent fibroblasts. We have shown that (i) membrane depolarization is due to activation of a new type of anion channel mediating net Cl^- efflux, with no apparent role for cation channels and (ii) serum action is attributable to albumin-bound LPA acting on its own G protein-coupled receptor, although it appears that serum contains other, as-yet-unidentified lipids with membrane-depolarizing activity for fibroblasts. That the depolarizing current is carried by Cl^- is based on the following findings: the current is enlarged by reducing extracellular Cl^-; unaffected by Na^+ removal; blocked by NFA; and associated with enhanced efflux of isotopic Cl^- and I^- (selectivity: $Cl^- > I^-$) but not Rb^+. If one reasonably assumes that the Cl^- equilibrium potential is close to the depolarization peak value (i.e. approximately –18 mV), then it follows from the Nernst equation that the intracellular Cl^- concentration in fibroblasts is about 80 mM. The Cl^- uptake mechanism responsible for maintaining cytosolic $[Cl^-]$ above equilibrium remains to be identified, but is likely to involve $Na^+/K^+/2Cl^-$ co-transport which operates in many cell types including fibroblasts (Lin and Gruenstein, 1988).

Our study adds a new cellular event to the growing list of biological activities of LPA and reinforces the notion that much of the biological activity of serum is due to platelet-derived LPA, rather than platelet-derived growth factor (PDGF). Previous studies have shown that albumin-bound LPA can account, at least in part, for such diverse serum effects as stimulation of phosphoinositide turnover in *Xenopus* oocytes and human carcinoma cells (Tigyi and Miledi, 1992; Eichholtz *et al.*, 1993; Seckl *et al.*, 1994), cytoskeletal changes as well as DNA synthesis in fibroblasts, enhanced fibronectin binding to adherent cells and neurite retraction in neuronal cell lines (for review see Jalink *et al.*, 1994; Moolenaar, 1995a,b).

Another new finding of the present study is that activation of the fibroblast Cl^- conductance is not unique for LPA, but is also observed with other G protein-coupled receptor agonists such as thrombin, endothelin and NKA

acting through their native or heterologously expressed receptors. It thus appears that induction of a depolarizing Cl^-current is a much more common reponse of activated fibroblasts than hitherto recognized. In this respect it should be mentioned that Cl^--mediated membrane depolarization has also been observed in smooth muscle-like cells stimulated with neuropeptides or neurotransmitters (Kremer *et al.*, 1989; Janssen and Sims, 1992; Van Renterghem and Lazdunski, 1993), where depolarization may promote Ca^{2+}-mediated contraction. As yet, we have been unable to record agonist-induced Cl^- currents at the single channel level. This may suggest that the channels have a very low conductance (<1 pS) and thereby escape detection. An alternative or additional possibility is that the agonist-induced Cl^- channels in adherent fibroblasts are physically inaccessible to patch–clamp electrodes: one such interesting situation would occur if the channels are localized predominantly at sites of cell–cell or cell–matrix interaction. We are currently investigating these possibilities.

Whatever the precise biophysical characteristics and membrane topology of the Cl^- channels, it appears that the newly found Cl^- conductance is distinct from the widely studied Cl^- channels activated by cell swelling, intracellular Ca^{2+} or cAMP. While current activation cannot be explained by known second messengers, we note that the depolarizing Cl^- current is invariably activated by receptors that couple to PTX-insensitive G proteins (G_q subfamily) to stimulate PLC. Furthermore, it is striking that channel activation coincides with Rho-mediated formation of focal adhesions and actin stress fibres, although we find that there is no direct cause–effect relationship between Rho activity and channel opening. Yet, in the simplest model compatible with the available data, Cl^- channel opening and Rho-mediated cytoskeletal changes are due to one and the same process, namely G_q-mediated inositol lipid hydrolysis. Indeed, the putative PLC inhibitor U-73122 blocks LPA-induced stress fibre formation (Chrzanowska-Wodnicka and Burridge, 1994) as well as Cl^- channel activation; (but note that the inhibitor by itself induced a depolarizing current in Rat-1 cells; see Results).

While our findings suggest that Cl^- channel activation is secondary to G_q-mediated activation of phosphoinositide-specific PLC (β-isotype), the response appears to be independent of phosphoinositide-derived messengers (Ca^{2+}, diacylglycerol, arachidonate metabolites). One possibility is that the PLC-mediated decrease in phosphatidylinositol-bisphosphate (PIP_2) levels somehow signals Cl^- channel opening as well as Rho activation; however, our findings leave open the possibility that PLC activation and Cl^- channel opening represent parallel rather than sequential events, with PLC and channel activation being regulated by distinct G protein subunits. Further studies, such as manipulating cellular PIP_2 levels and elucidating the putative PLC–Rho connection, will help address how Cl^- channel activation is linked to the G_q-PLC pathway.

There is currently much interest in Cl^- channels, as they serve important cellular functions in volume regulation, modulation of membrane excitability and epithelial fluid secretion, while inherited defects of Cl^- channels may lead to severe diseases (Welsh and Smith, 1993; Jentsch, 1994; Hofmann *et al.*, 1995). Cl^- channel activation as described here for fibroblasts apparently serves to depolarize the plasma membrane. What, then, are the voltage-sensitive membrane process(es) driven or modulated by the observed membrane depolarization? Among the best known and most voltage-sensitive processes are activation of voltage-gated ion channels. In addition to nerve and muscle cells, some fibroblasts also express voltage-sensitive Ca^{2+} channels (Chen *et al.*, 1988). On the other hand, membrane depolarization reduces the driving force for Ca^{2+} entry (through voltage-independent channels) and thus may serve to attenuate Ca^{2+} signalling (for example see DiVirgilio *et al.*, 1987). Besides having a possible role in cell signalling, the membrane potential can act electrophoretically and, as such, may promote (or impede) the translocation of charged protein domains across the plasma membrane (Cao *et al.*, 1995; Otero *et al.*, 1995). There is also evidence for a regulatory role of membrane depolarization on cytokine release (Haslberger *et al.*, 1992). Clearly, elucidation of the function of sustained membrane depolarization in quiescent fibroblasts awaits further studies.

Materials and methods

Materials

Bis-oxonol (cat no. B-413) and TRITC-labelled phallacidin were from Molecular Probes (Eugene, OR, USA). Genistein, stauroporine, herbimycin A, tyrphostin 25, $AACOCF_3$ and U-73122 were from Calbiochem. LPA (1-oleoyl and 1-palmitoyl) and other lipids, endothelin, NKA and all other chemicals were from Sigma. TRP (sequence: SFLLRNPND-KYEPF) was synthesized as described (Jalink and Moolenaar, 1992). $H^{36}Cl$ (19 μCi/mg); $Na^{125}I$ (14 mCi/mg) and $^{86}RbCl$ (5 mCi/mg) were from Amersham. Recombinant C3 transferase was kindly provided by Shuh Narumiya (Kyoto University, Japan). MK-866 was a gift from Merck Frosst Inc. (Quebec, Canada) and Zileuton from Abbott Laboratories (Illinois, USA).

Cell culture

Rat-1 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. Cultures were rendered quiescent by exposing them to serum-free DMEM for 2–3 days prior to experimentation.

Electrophysiology

Electrophysiological responses of serum-deprived Rat-1 cells were measured using either intracellular or whole-cell patch–clamp recordings at room temperature. Cells were grown in 3-cm plastic dishes. It was verified that results obtained at room temperature were not significantly different from those obtained at 37°C. Data were collected using an EPC-7 amplifier (List-Medical), interfaced to a personal computer via an A/D converter (TL-1 DMA interface, Axon Instruments Inc.). Voltage–clamp protocols were generated and data were stored using pClamp 5.5 or Axotape 2.0 (Axon Instruments Inc.). Recordings were carried out using the perforated patch–clamp technique with amphotericin in the pipette solution (to selectively permeabilize the membrane to monovalent ions), as described by Rae *et al.*, (1991). Borosilicate glass capillaries (Clark electromedical instruments 1.5mm O.D ×1.17 mm I.D) were pulled on a computerized Brown-Flaming type pipette puller (Sutter Instrument Co, model p-87) and fire polished using a microforge (Narishige Co, Japan, model mf-9). Pipettes were filled with a solution containing: 120 mM KGlu, 30 mM KCl, 10 mM HEPES (pH 7.2), 0.2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM EGTA, 30 mM mannitol and 240 μg/ml amfotericin B. The composition of the extracellular medium was: 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4). Osmolarity of all media was adjusted to 310 mOsm using a cryoscopic osmometer.

Prior to establishing a seal, pipettes typically showed a resistance of 3–5 MOhm. After establishing a seal (GOhm), series resistance dropped due to the permeabilizing action of amfotericin B to a steady value of 13.1 ± 1.7 MOhm (usually within 10 min in single cell recordings). At

this point, stable membrane potentials could be measured and experiments were initiated. Series resistance and membrane capacitive transients could not be compensated for, due to electrical coupling of the cells; however, agonist-induced currents were relatively small and showed slow kinetics, and therefore this was not considered a problem. Agonist and pharmacological inhibitors were applied either manually or by micro-perfusion. All experiments are done at least in triplicate; results are given as the mean ± SEM and were analyzed using the Student's *t*-test.

Bis-oxonol fluorimetry

Agonist-induced changes in membrane potential were monitored in 80–100% confluent cell cultures (grown on glass coverslips) either by cuvette measurements in a Perkin-Elmer model LS-3B fluorescence spectrometer or by confocal microscopy (Bio-Rad) using bis-oxonol as a potential-sensitive probe. Bis-oxonol was added to the cells at a final concentration of 200 nM. The dye was allowed to equilibrate with the cells for 10–15 min before beginning an experiment. Bis-oxonol fluorescence (F) was monitored at an excitation wavelength of 540 nm and emission wavelength of 580 nm. For calibration purposes and to assess cell viability, at the end of each experiment, 1 μM gramicidin was added to induce collapse of the membrane potential. Although measurements with bis-oxonol do not allow quantitative estimates of membrane potential values and kinetics, and are prone to fluorescence artefacts, they are technically much less demanding than electrophysiological experiments and very convenient for assaying the effects of agonists, inhibitors, etc.

Efflux assays

Confluent monolayers of Rat-1 cells in 6-well culture plates were loaded with 5 μCi of either $^{125}I^-$ or $^{36}Cl^-$ and 0.5 μCi of $^{86}Rb^+$ for 2 h and washed ×3 with isotonic buffer (composition: 80 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 95 mM mannitol and 20 mM HEPES, pH 7.4) prior to the assay. Isotope efflux was determined at 37°C by replacing the medium at 1.5 min intervals. Bumetanide (100 μM) was present to inhibit cation/Cl^- co-transport. Hypotonic buffers were prepared by adjusting the concentration of mannitol. Radioactivity in the medium was determined by gamma radiation counting and expressed as fractional efflux per min as previously described (Vaandrager *et al.*, 1991). The initial increase in fractional anion effluxes measured at the earliest time point after agonist addition (1.5 min) was considered the most representative indicator of the effects of the agonists on anion efflux.

Inositol phosphate measurements

Accumulation of total inositol phosphates in agonist-stimulated Rat-1 cells was measured in the presence of 10 mM LiCl, as described by Alblas *et al.* (1995).

Detection of actin stress fibres

Cells were fixed in 3% paraformaldehyde and permeabilized in 0.2% Triton X-100. Actin filaments were visualized using TRITC-labelled phallacidin as previously described (Jalink and Moolenaar, 1992). Agonist-induced formation of actin stress fibres in serum-starved cells was compared to untreated control cells and scored semi-quantitatively using a Zeiss Axiophot fluorescence microscope or a Bio-Rad confocal microscope.

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