JOURNAL OF CELLULAR PHYSIOLOGY 170:166–173 (1997)

Membrane Depolarization in NRK Fibroblasts by Bradykinin Is Mediated by a Calcium-Dependent Chloride Conductance

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The effects of the phosphoinositide-mobilizing agonist bradykinin (BK) on membrane potential and intracellular calcium in monolayers of normal rat kidney (NRK) fibroblasts were investigated. BK induced a rapid transient depolarization in these cells, which was mimicked by other phosphoinositide-mobilizing factors such as prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), lysophosphatidic acid (LPA), platelet-derived growth factor (PDGF-BB), and serum. Depolarization by BK was independent of extracellular Ca^{2+} or Na^+. It was shown using extracellular Cl^- substitutions that the depolarization was caused by an increased Cl^- conductance. Depolarization was inhibited by 5-nitro-2-3-phenylpropyl(amino)benzoic acid (NPPB), niflumic acid, and flufenamic acid, inhibitors of calcium-dependent chloride channels. The depolarization provoked by BK could be mimicked by raising intracellular calcium with ionomycin or thapsigargin and could be blocked with geneticin, a blocker of phospholipase C. When intracellular calcium was buffered by loading the cells with 1,2-bis(2-aminophenoxy)ethane-NNN'N'-tetra-acetic acid (BAPTA), depolarization was prevented. We conclude that in NRK fibroblasts extracellular stimuli that increase intracellular calcium, depolarize the cells via the activation of a calcium-dependent chloride conductance. In addition to an increase in intracellular calcium, depolarization may be an important effector pathway in response to extracellular stimuli in fibroblasts. It is hypothesized that, in electrically coupled cells such as NRK fibroblasts, intercellular transmission of these depolarizations may represent a mechanism to coordinate uniform multicellular responses to Ca^{2+}-mobilizing agonists. **J. Cell. Physiol. 170:166–173, 1997.** © 1997 Wiley-Liss, Inc.

The phosphoinositide-mobilizing agonist bradykinin (BK) is a peptide exerting a wide variety of pharmacological and physiological activities in different cell types. The receptors of BK belong to the class of G-protein-coupled receptors possessing seven membrane-spanning domains, which, upon binding of their ligand, activate, among others, phospholipase C with consequent Ca^{2+}-mobilization from internal stores by released inositolphosphates and activation of protein kinase C by diacylglycerol.

Biological effects caused by BK include vasodilatation, altered ion transport, induction of inflammatory processes, smooth muscle contraction, and modulation of cell proliferation (Roberts, 1989). Many of these processes are mimicked by other phosphoinositide-mobilizing agents like prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Negishi et al., 1993; Watanabe et al., 1994). BK also has been shown to inhibit the loss of density-dependent growth inhibition by transforming growth factor β (TGFβ) or retinoic acid in normal rat kidney (NRK) fibroblasts (van Zoelen et al., 1994). This effect of BK is not mimicked by $PGF_{2\alpha}$ and is mediated by a so far unknown second messenger, probably a prostaglandin derivative (Afink et al., 1994; Lahaye et al., 1994).

Apart from calcium release and PKC activation, BK has been shown to induce arachidonic acid release (van Corven et al., 1989; Afink et al., 1994), prostaglandin synthesis (Regoli and Barabe, 1980), and tyrosine phosphorylation (Coutant et al., 1995). BK also has been shown to have several effects on ion conductances and membrane potential, depending on the cell type (Higashida and Brown, 1986; Fasolato et al., 1988; Mehrke and Daut, 1990; Clarke et al., 1992; Pavenstädt et al., 1993). Although fibroblasts express receptors for many extracellular stimuli, including BK, that increase intracellular calcium by a release of inositolphosphates (van Corven et al., 1989; Afink et al., 1994; van Zoelen et al., 1994; Postma et al., 1996), little is known about the electrophysiological effects of these agonists in these cells.

To gain more insight in the electrophysiological phenomena caused by BK and $PGF_{2\alpha}$ in fibroblasts, we studied their effects on both membrane potential and

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Received 19 June 1996; Accepted 30 September 1996

intracellular calcium in monolayers of NRK cells that are known to express receptors for both agonists (Afink et al., 1994). BK, $PGF_{2\alpha}$, and other phosphoinositide-mobilizing agonists induced a rapid, transient depolarization, concomitant with a rise in intracellular calcium. We show that this depolarization results from the activation of a calcium-activated chloride conductance. Membrane depolarization in response to an increase in intracellular calcium could be an important effector pathway in the signaling of calcium-mobilizing agonists in fibroblasts.

MATERIALS AND METHODS

Cell culture

NRK fibroblasts (clone 49F) were seeded at a density of $1.0 \cdot 10^4$ cells/cm^2, and grown to confluence in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% newborn calf serum. Confluent cells were made quiescent by a subsequent 1- to 3-day incubation in serum-free DF medium (DMEM/Ham's F12, 1:1; Gibco, Life Technologies, Paisley, U.K.) supplemented with 30 nM Na_2SeO_3 and 10 μg/ml human transferrin. When density-arrested cells were used, confluent serum-free cultures were treated for an additional 48 hr with 5 ng/ml EGF (Collaborative Biomedical Products, Bedford, MA) and 5 μg/ml insulin (Sigma, St. Louis, MO), as described previously (van Zoelen et al., 1988).

Voltage and current clamp measurements

For whole-cell patch-clamp studies, cells were perfused at a rate of 1–2 ml per minute with serum-free DF medium (5% CO_2) at room temperature. DF medium is a cell culture medium that contains as main inorganic salts (in mM): 109.5 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.81 $MgCl_2$, 44.0 $NaHCO_3$, and 1.0 NaH_2PO_4, and is supplemented with essential nutrients such as glucose, amino acids and vitamins for optimal cell growth. Convential whole-cell patch-clamp methods were used. Pipettes were filled with a high K^+, Tris-buffered solution (in mM: 25 NaCl, 120 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Tris, and 3.5 Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), pH 7.4). An EPC-7 patch-clamp amplifier (List, Darmstadt, Germany) and CED software (Cambridge Electronic Design Limited, Cambridge, U.K.) were used to acquire data. Acquisition was obtained at a rate of 50 Hz in both the current-clamp and voltage-clamp measurements and digitally filtered at 10 Hz. In ion substitution experiments, DF-medium was made from scratch, and sodium or potassium was replaced by *N*-methyl-D-glucamine and chloride was replaced by gluconate. In calcium-free experiments, calcium chloride was omitted from the medium and 1 mM EGTA was added. Normal DF medium contains 127 mM Cl^-.

Intracellular calcium measurements

For calcium measurements, cells were seeded on gelatin-coated glass coverslips and cultured as described above. Quiescent monolayers were loaded with 2 μM Fura-2AM with 0.01% pluronic acid for 30 min at room temperature, washed for 15–30 min in serum-free medium, and then placed in a modified Leiden perfusion chamber. For loading the cells with 1,2-bis-(o-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid acetoxymethyl ester (BAPTA-AM) the same protocol was used as for Fura-2AM. Cells were perfused with DF medium at a rate of 1 ml/min at room temperature. Excitation wavelengths was 340 nm, whereas emission was detected at 510 nm. Fluorescence was detected using a photon counting microspectrofluorimetric system (Newcastle Photometric System Ltd, Newcastle, U.K.). In combined patch-clamp and calcium measurements this photometric system was combined with an EPC-9 patch-clamp setup, which was not basically different from the patch-clamp setup described above.

Chemicals

$PGF_{2\alpha}$, indomethacin, forskolin, thapsigargin, pertussis toxin, niflumic acid, flufenamic acid, ionophore A23187, melittin, arachidonic acid, and ionomycin were from Sigma (St. Louis, MO); geneticin, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), BK, geneticin, and genistein were from Boehringer (Mannheim, Germany); 5-nitro-2-3-phenylpropyl(amino)benzoic acid (NPPB) and diphenylamine-2-carboxylic acid (DPC) were from RBI (Natick, MA); and 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS), BAPTA-AM, and Fura-2AM were from Molecular Probes (Eugene, OR). Arachidonic acid was from Sigma and was 1,000 times diluted in DF-medium from a stock solution of 1 mg/ml in ethanol before use. Platelet-derived growth factor (PDGF-BB) was from Genzyme (Cambridge, MA), while TGFβ was purified from human platelets as described (van den Eijnden-van Raaij et al., 1988).

RESULTS

Bradykinin depolarizes NRK fibroblasts

The effect of BK on membrane potential was investigated using the current-clamp mode of the whole-cell patch-clamp technique. Figure 1A shows that BK (100 nM) caused a rapid depolarization of the membrane in serum-deprived quiescent NRK cells. The depolarization slowly recovered and typically lasted up to 5–10 min. This effect could be mimicked by $PGF_{2\alpha}$ (Fig. 1A). Depolarization occurred within 30 sec after BK was perfused to the bath and membrane potential dropped from an initial value of -71.2 ± 3.4 mV (mean ± SD; $n = 44$) to a value of -16.7 ± 2.2 mV ($n = 15$). Depolarization induced by $PGF_{2\alpha}$ was not significantly (-19.0 ± 3.5 mV; $n = 5$) different (Fig. 1B). Repolarization was faster when lower (10 nM and less) concentrations of the agonists were used. Similar results were obtained when density-arrested cells were used (data not shown). Addition of other phosphoinositide-mobilizing factors like PDGF-BB (10 ng/ml), LPA (5 μM), or serum (2%) also depolarized the cells. On the other hand, EGF and TGFβ, growth factors that do not cause phosphatidylinositol turnover in NRK fibroblasts (A.D.G. de Roos, A.P.R. Theuvenet, and E.J.J. van Zoelen, unpublished results), did not depolarize the cells. Thus, typically agonists that share the ability to activate phospholipase C cause a depolarization of NRK fibroblasts.

After washout of either BK or $PGF_{2\alpha}$ and repolarization of the cells, a second dose of the same agonist did not induce a second depolarization. On the other hand, prior exposure to BK did not attenuate the response to $PGF_{2\alpha}$ and vice versa (data not shown). This shows that the response to BK and $PGF_{2\alpha}$ exhibits homologous, but no heterologous desensitization.

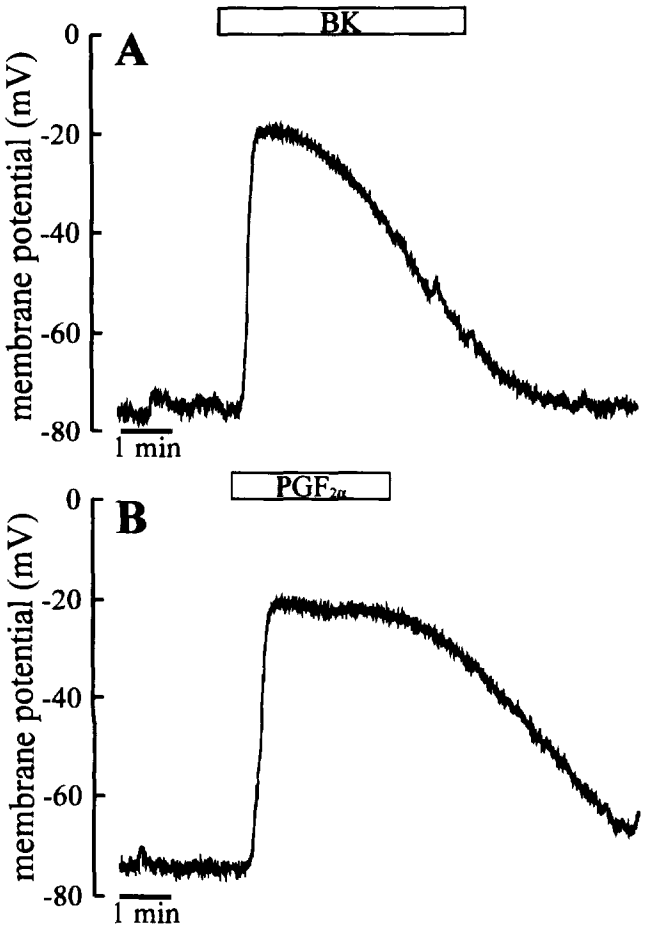

Fig. 1. Effects of BK and $PGF_{2\alpha}$ on membrane potential in monolayers of NRK fibroblasts. **A:** Typical depolarization in response to 100 nM BK measured in the current-clamp mode of the whole-cell patch-clamp configuration. **B:** Depolarization in response to 100 nM $PGF_{2\alpha}$. Cells were continuously perfused and the agonists were included in the perfusion medium during the indicated time. Each trace is representative of at least 10 similar experiments.

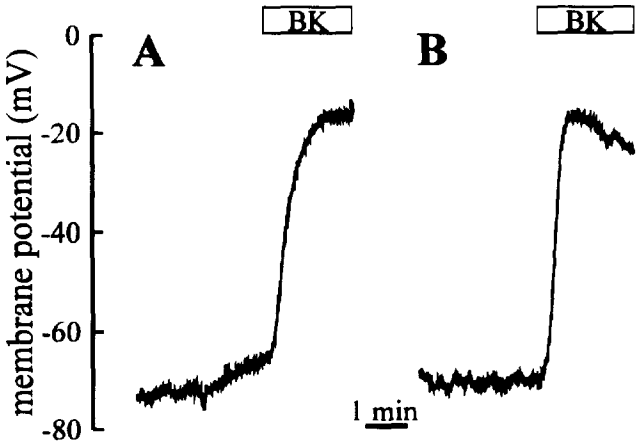

Fig. 2. Role of calcium influx in the depolarization by BK. **A:** Effect of 100 nM BK in a nominal calcium-free medium, supplemented with 1 mM EGTA. **B:** Response to BK in the presence of 1 μM felodipine. Similar results were obtained with 100 nM $PGF_{2\alpha}$. Each trace is representative of at least four similar experiments.

BK induces a chloride conductance

To determine which inward currents could be responsible for the depolarization by BK, ion substitutions of the perfusion medium were performed. We recently showed that monolayers of NRK cells are electrically well coupled (de Roos et al., 1996). This is demonstrated by the fact that the total membrane conductance was in the low MΩ range and that changing pipette medium to an extracellular medium had no effect on measured membrane potential in monolayers of NRK cells. This shows that there was electrical access to the other cells in the monolayer via the patched cell and that an average membrane potential over the whole monolayer was measured. We, therefore, were limited to changes in the extracellular solution to determine which ions carry the current.

Lowering the extracellular sodium concentration to 5 mM had no effect on the depolarization, nor on resting membrane potential. Also, 5 min of perfusion with a calcium-free medium (containing 1.0 mM EGTA with no calcium added) did not affect the depolarization by BK (Fig. 2A). Also, when the concentration of sodium ions was lowered in a calcium-free medium, the depolarization was not affected (not shown), excluding a possible contribution of sodium influx through calcium channels to the depolarization when no calcium is present. Moreover, Fig. 2B shows that the L-type calcium channel blocker felodipine did not affect the depolarization by BK. These results show that an increased permeability for sodium or calcium ions is not involved in the depolarization. However, lowering the extracellular chloride concentration to 8 mM, and thereby reversing the concentration gradient for chloride, had a remarkable effect on the depolarization without a change in the resting potential. Figure 3A shows that, when the medium was changed to a low chloride medium after depolarization by BK, the membrane potential shifted to a value as high as +20 mV, indicating that the depolarization is mediated by a chloride conductance. Moreover, when the cells were first perfused with a low Cl^- medium (Fig. 3B), the membrane potential changed after the addition of BK from an initial value around −70 mV to positive values, according to a positive equilibrium potential for Cl^- when the concentration of extracellular Cl^- is lower than the intracellular concentration. Under these conditions, BK induced also a fast action potential-like spike that preceeded the depolarization. The ability of BK to generate action potentials is also seen in media in which the calcium gradient is increased and has been shown to result from the regenerative opening of voltage-dependent calcium channels (A.D.G. de Roos et al., unpublished results).

Figure 3C shows the response to BK when the membrane was first depolarized by 125 mM potassium. Bradykinin caused here a change in membrane potential toward an expected, negative, equilibrium potential for Cl^-. Addition of BK to cells that were depolarized by 125 mM K^+ in a low Cl^- medium (Fig. 3D) also shifted the membrane potential to the expected, now positive, equilibrium potential for Cl^-. These effects were all

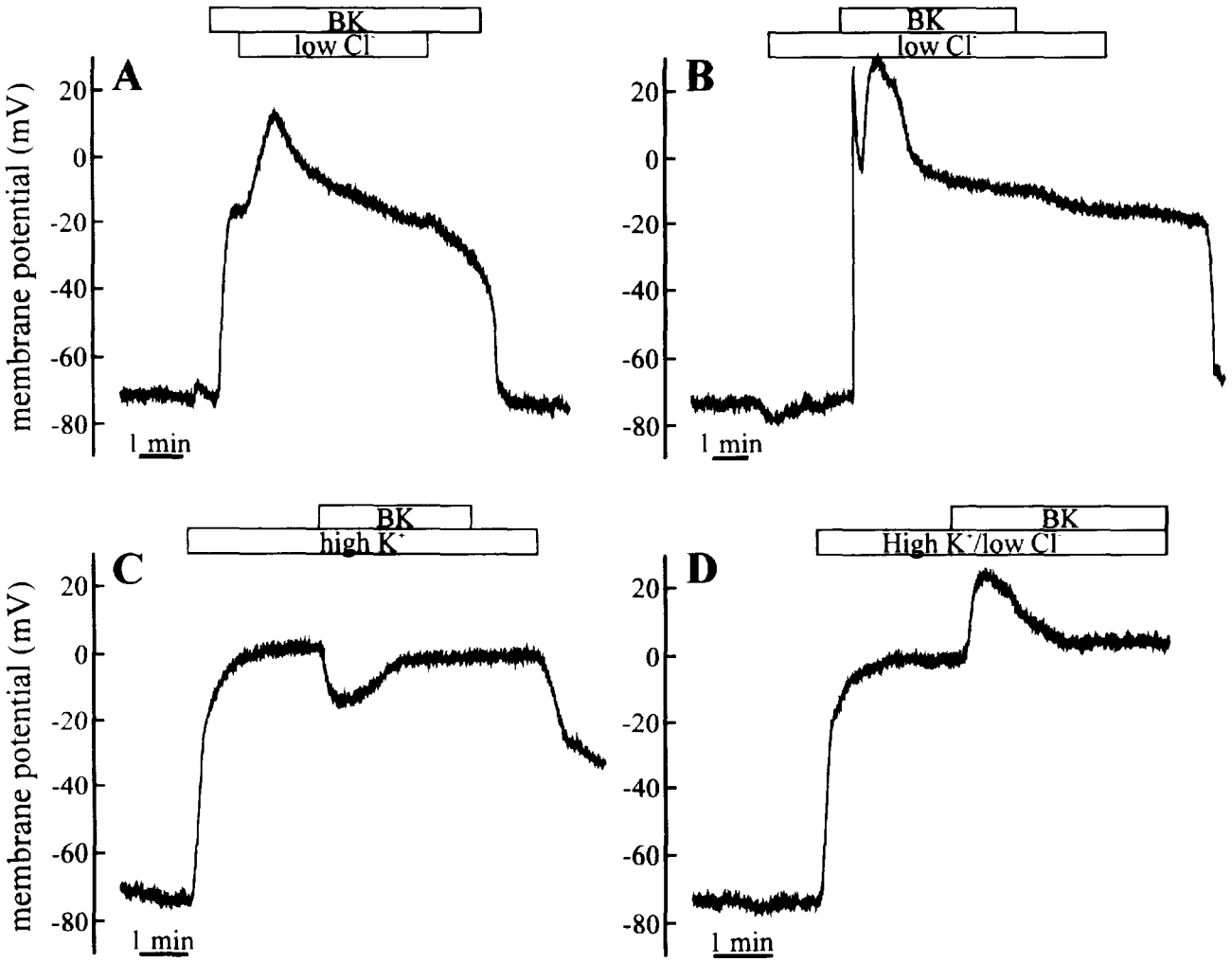

Fig. 3. Cell membrane potential responses to BK in solutions of varying ionic composition. **A:** Effect of changing to a solution that contained 8 mM Cl^- after the cells were depolarized with 100 nM BK. **B:** Response to BK in a solution containing 5 mM Cl^-. **C:** Response to 100 nM BK after the cells were first depolarized with a medium that contained 120 mM K^+. **D:** Effect of BK (100 nM) in a medium with 120 mM K^+ (high K^+) and 8 mM Cl^- (low Cl^-). Similar results were obtained with 100 nM $PGF_{2\alpha}$. Each trace is representative of at least four similar experiments.

mimicked by $PGF_{2\alpha}$. We conclude that the depolarization by both BK and $PGF_{2\alpha}$ is caused by an increased chloride conductance.

Effect of second messenger modulators and channel blockers

BK and $PGF_{2\alpha}$ have been reported to affect various second messenger pathways (van Corven et al., 1989; Afink et al., 1994). Therefore, we tested several modulators of these pathways. The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C (PKC), transiently inhibits electrotonic coupling between NRK cells, but also is able to depolarize the cells (de Roos et al., 1996). Cells in which PKC was downregulated by prolonged treatment (24–48 hr) with 100 ng/ml TPA were used to investigate whether the depolarization by BK and $PGF_{2\alpha}$ was mediated by PKC. Membrane potential in PKC-downregulated cells was similar to control cells, and both BK and $PGF_{2\alpha}$ were still able to depolarize these PKC-downregulated cells, whereas TPA had no effect. This shows that the depolarization by BK and $PGF_{2\alpha}$ is not mediated by PKC.

Inhibition of G_i-proteins by pertussis toxin (100 ng/ml; 3-h preincubation), inhibition of tyrosine phosphorylation by genistein (50 μM; 10 min preincubation), activation of phospholipase A_2 by melittin (8 μg/ml) and inhibition of cyclooxygenase by indomethacin (10 μM, 60 min preincubation) failed to block the depolarization process (results not shown). Also, depolarization could not be mimicked by forskolin (10 μM) or arachidonic acid (1.0 μg/ml). We also tested several chloride channel blockers (Greger, 1990; White and Aylwin, 1990; Frizzell and Morris, 1994) to identify the chloride channel involved. DPC (250 μM) and DNDS (100 μM) did not block the response (not shown), but NPPB (50 μM), flufenamic acid, and niflumic acid (250 μM), all three

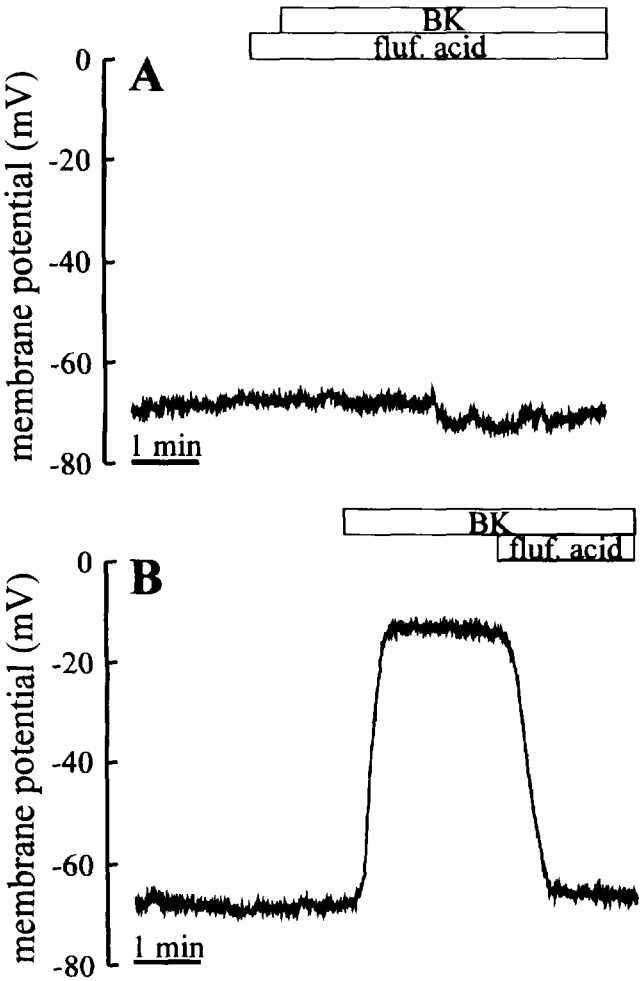

Fig. 4. Flufenamic acid blocks the depolarization evoked by BK. **A:** The effect of BK (100 nM) on the membrane potential after perfusion of 250 μM flufenamic acid. **B:** Flufenamic acid (250 μM) quickly reverses the depolarization caused by BK. Similar results were obtained using niflumic acid (250 μM) instead of flufenamic acid and $PGF_{2\alpha}$ (100 nM) instead of BK. Each trace is a typical result of at least four similar experiments.

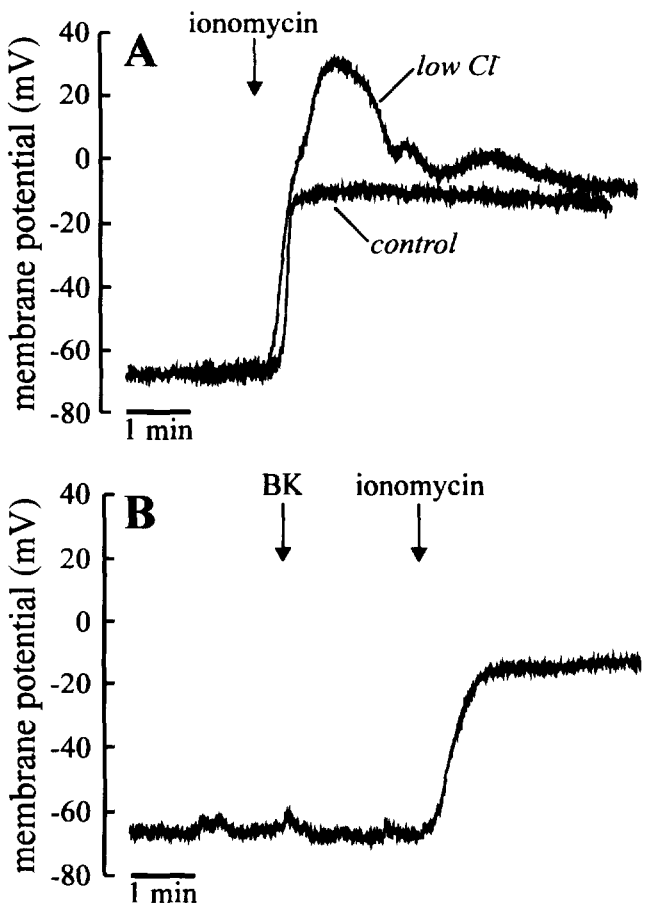

Fig. 5. Effect of modulation of intracellular calcium on membrane potential. Ionomycin (0.5 μM; **A**) induces a depolarization (control) that was enhanced in 8 mM Cl^- (low Cl^-) **B:** The PLC inhibitor geneticin (0.5 mg/ml) blocks the response to 100 nM BK, but not that to ionomycin (5 μM). The cells were preincubated for 30 min with geneticin. Ionomycin and BK were added directly to the bath. Each trace is a typical result of at least four similar experiments.

calcium-activated chloride channel blockers (Greger, 1990; White and Aylwin, 1990) were able to block the depolarization completely (Fig. 4A). When flufenamic acid or niflumic acid was perfused to the cells after the depolarization by BK, the membrane repolarized quickly to resting levels (Fig. 4B), showing that the block by niflumic acid and flufenamic acid is fast and that these blockers act directly on the chloride channel involved. The fact that the depolarization could be prevented and reversed by calcium-activated chloride channel blockers suggests a role of calcium.

Depolarization is calcium-mediated

It is known that BK, but also $PGF_{2\alpha}$, can release calcium from intracellular stores in NRK fibroblasts (Afink et al., 1994; A.D.G. de Roos, unpublished observation). To further investigate the possible role of calcium in activation of the chloride conductance, we tried to mimic the BK-induced depolarization by increasing intracellular calcium using the ionophores ionomycin and ionophore A23187 and the endoplasmic reticulum calcium ATPase inhibitor thapsigargin. It was confirmed by Fura2 fluorescence measurements that these agents increased intracellular calcium in NRK cells (data not shown). Ionomycin (5 μM) induced a depolarization (Fig. 5A) similar to BK and $PGF_{2\alpha}$ that also was observed by 10 μM ionophore A23187 or 1.0 μM thapsigargin (data not shown). Figure 5A also shows that the depolarization by ionomycin was potentiated by lowering extracellular Cl^-, showing that ionophore activates the same conductive pathway as BK and that the depolarization by the relatively high concentration of ionomycin does not result from a general permeabilization of the plasma membrane. Figure 5B shows that geneticin (0.5 mg/ml), a blocker of phospholipase C (Rock and Jackowski, 1987), was able to block the response to BK but not that to ionomycin. These findings suggest that the block of the response to BK is due to the inhibition of the release of inositolphosphates by

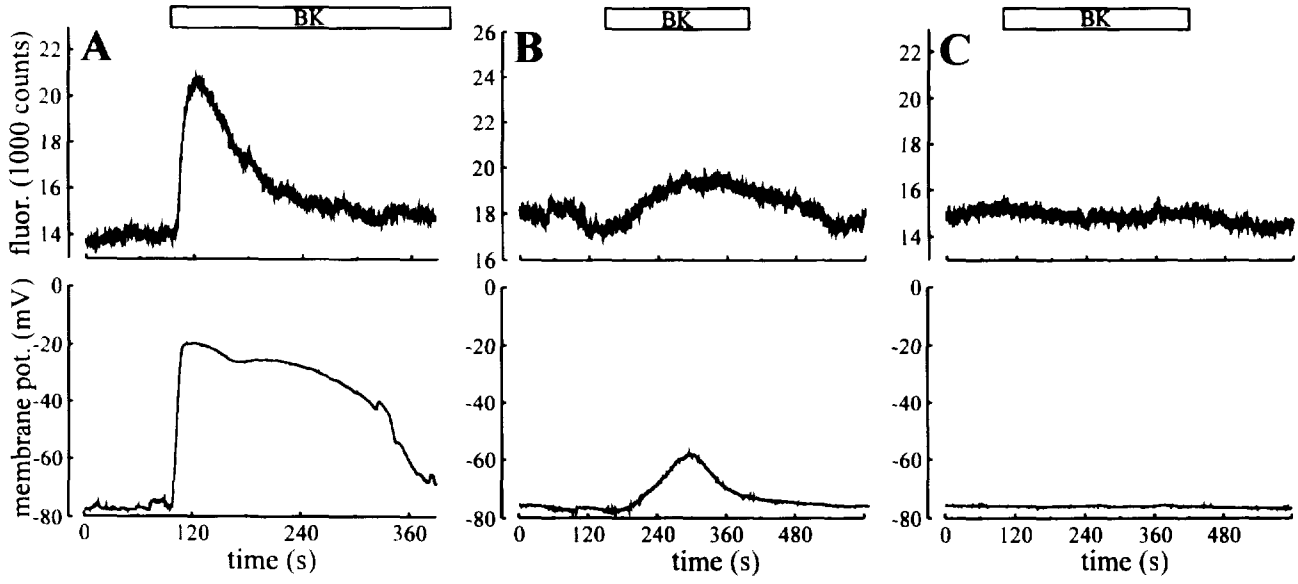

Fig. 6. Intracellular calcium buffering by BAPTA. Intracellular calcium (top) was recorded simultaneously with the membrane potential (bottom). **A:** The calcium and membrane potential response in cells that were not loaded with BAPTA. **B** and **C:** The responses in cells that were loaded with BAPTA by incubating them with 5 (B) and 10 μM (C) BAPTA-AM. NRK cells were incubated with indicated concentrations of BAPTA-AM and Fura-2AM for 30 min as described in Materials and Methods. After a 15-min wash, a cell was patched in the whole-cell configuration to measure the membrane potential, whereas calcium was determined photofluorimetrically. The calcium signal represents the average of about 20 cells in the monolayer. Each trace is representative of at least three similar experiments.

geneticin, thereby preventing calcium release from intracellular stores. These results support the notion that a rise in cytosolic calcium is sufficient to activate the chloride conductance and to depolarize NRK cells.

To further investigate the role of cytosolic calcium in relation to the depolarization, patch-clamp measurements were performed simultaneously with spectrofluorimetric measurements of intracellular calcium. Figure 6A shows that the onset of the increase in intracellular calcium (top panel) correlates well with the depolarization (lower panel), indicating that these are closely related. A buffering of intracellular calcium was achieved by loading the cells with the acetoxymethyl ester of BAPTA (BAPTA-AM). By the action of aspecific esterases in the cell, the acetoxymethyl group can be cleaved and the calcium chelator BAPTA will be trapped in the cell. Figure 6, B and C, shows the response in intracellular calcium and membrane potential to BK and $PGF_{2\alpha}$ when the cells were loaded with BAPTA, by incubating them with 5 and 10 μM BAPTA-AM, respectively. Figure 6B shows that after loading the cells with 5 μM BAPTA, both the increase in intracellular calcium and membrane depolarization in response to BK were diminished. The cells still could be depolarized by 120 mM K^+ (not shown) after loading with BAPTA, indicating that BAPTA does not have an aspecific effect on ion conductances. Loading the cells with 10 μM BAPTA completely abolished both the depolarization and the increase in intracellular calcium (Fig. 6C). Thus, prevention of an increase in intracellular calcium also prevented the depolarization, showing that the depolarization is calcium-dependent. Inhibition of the depolarization, however, by niflumic acid did not impair the ability of BK and $PGF_{2\alpha}$ to increase intracellular calcium (results not shown).

Taken together, the results show that depolarization of NRK cells by the phosphoinositide-mobilizing agonists BK and $PGF_{2\alpha}$ is the result of activation of a calcium-dependent chloride channel.

DISCUSSION

The phosphoinositide-mobilizing agent BK has been shown to have a wide range of effects depending on cell type. Short-term effects of BK include mobilization of calcium from intracellular stores, activation of PKC, release of arachidonic acid, and tyrosine phosphorylation (Roberts, 1989; Coutant et al., 1995). Here, we showed that BK causes a rapid and transient depolarization in NRK fibroblasts that was mimicked by $PGF_{2\alpha}$ (Cole et al., 1988; Negihsi et al., 1993). We presented evidence that this depolarization is caused by a calcium-activated chloride current. i) Using ion substitutions, a change in the extracellular Cl^- but not Ca^{2+} or Na^+ concentration had an effect on the magnitude of the depolarization. ii) Depolarization could be blocked and reversed by the calcium-activated chloride channel blockers NPPB, niflumic acid, and flufenamic acid. iii) Other phosphoinositide- and calcium-mobilizing factors such as LPA, PDGF-BB, and serum also depolarized the cells. Thapsigargin and the calcium ionophores ionomycin and A23187, substances that increase intracellular calcium, were able to mimic the depolarization by BK and $PGF_{2\alpha}$. Flufenamic acid and niflumic acid were able to block this response. Also, inhibition of phospholipase C by geneticin blocked the depolarization by BK. iv) The kinetics of depolarization and the increase in intracellular calcium were similar and the BK-induced depolarization was blocked when intracellular calcium was buffered with BAPTA. We conclude that the depolarization by BK and other phosphoinosi-

tide-mobilizing agents, such as $PGF_{2\alpha}$, is the result of the activation of a calcium-dependent chloride conductance.

Our results show that an increase in intracellular calcium is sufficient to depolarize NRK cells. Growth factors that do not increase intracellular calcium in NRK cells, such as EGF, which acts via a tyrosine kinase receptor, or TGFβ, which activates a serine/threonine kinase receptor, had no effect on membrane potential in NRK cells. Calcium-mobilizing agonists have been shown to induce a variety of electrophysiological effects, depending on the cell type. For example, BK has been shown to activate a Na^+ and Cl^- conductance in human nasal epithelium (Clarke et al., 1992) and causes a hyperpolarization followed by a depolarization in neuronal (Higashida and Brown, 1986), epithelial cells (Pavenstädt et al., 1993), endothelial (Mehrke and Daut, 1990) and PC12 cells (Fasolato et al., 1988). A hyperpolarization in these cells is primarily the result of activation of calcium-activated potassium channels, whereas a depolarization has been attributed to inhibition of voltage-dependent potassium channels (Higashida and Brown, 1986), to a Cl^- current (Pavenstädt et al., 1993), and to a cation influx (Fasolato et al., 1988). Nonselective cation conductances have been implicated in agonist-induced depolarization of fibroblasts by PDGF (Frace and Gargus, 1989). Recently, it has been found that lysophosphatidic acid (LPA) depolarizes quiescent Rat-1 fibroblast by activation of a Cl^- conductance. This conductance also was related closely to PLC activation, but reported to be independent of known second messengers including Ca^{2+} (Postma et al., 1996). This shows that activation of Cl^- channels may be a common mechanism by which G-protein-coupled receptors exert their action in fibroblasts.

Using ion substitutions, we showed that the depolarization evoked by BK was carried by chloride ions. Assuming that the peak of depolarization (−17 mV) corresponds with the equilibrium potential for chloride ions, it follows from the Nernst equation that the intracellular Cl^- concentration is approximately 65 mM (extracellular 127 mM Cl^-). This is in good agreement with the concentration found in other fibroblasts, which ranges from 70 to 87 (Lamb and MacKinnon, 1971; Chao et al., 1989; Leeves and McDonald, 1995; Postma et al., 1996). This high intracellular Cl^- concentration probably is maintained by a Cl^-/HCO_3^- exchanger and $Na^+/K^+/2Cl^-$ cotransporter (Lin and Gruenstein, 1988; Chao et al., 1989).

Depolarization by BK and $PGF_{2\alpha}$ was independent of extracellular Ca^{2+} but was abolished by preloading the cells with BAPTA, showing that the activation of the chloride conductance required mobilization of calcium from intracellular stores. Whether intracellular calcium activates this conductance directly or through a calcium/calmodulin complex or calcium/calmodulin-dependent kinases, as suggested in the activation of a calcium-dependent chloride conductance in epithelia (Frizzell and Morris, 1994), is yet unclear.

NRK cells contain L-type voltage-dependent channels (de Roos et al., unpublished data), which is illustrated by the fact that depolarization with KCl induces an increase in intracellular calcium, which is blocked by felodipine (not shown). Therefore, it is expected that the depolarization by BK also activates calcium channels, which may contribute to the activation of calcium-dependent chloride channels. However, the fact that felodipine did not affect the depolarization by BK (Fig. 2B) suggests that opening of L-type calcium channels does not play a role in the depolarization by BK.

So far, we have not been able to see any activation of chloride channels by patch-clamp measurements in cell-attached or excised patches. This may suggest that the channel density is very low or that very low-conductance Cl^- channels are activated. The involvement of low-conductance calcium-activated Cl^- channels has been suggested in HT29 cells (Greger and Kunzelmann, 1991). Also, in monolayers of Rat-1 fibroblasts, no agonist-induced Cl^- currents could be detected at the single channel level, and it has been hypothesized that the Cl^- channels involved might be localized predominantly at the sites of cell-cell or cell-matrix contacts, and are therefore inaccessible to patch-clamp electrodes (Postma et al., 1996). This might apply to monolayers of NRK fibroblasts as well.

In NRK fibroblasts, the resting membrane potential is close to the expected equilibrium potential for potassium ions. Therefore, large hyperpolarizations due to opening of calcium-activated K^+ channels as reported in other cells (Higashida and Brown, 1986; Fasolato et al., 1988; Pavenstädt et al., 1993) are not likely. Even small hyperpolarizations after addition of the potent calcium-mobilizing agents BK or $PGF_{2\alpha}$ were never seen in NRK cells. This indicates that the activation of K^+ channels by a rise in intracellular calcium does not play a role in the modulation of the membrane potential of NRK fibroblasts. It is possible, though, that NRK cells possess voltage-dependent K^+ channels that may facilitate the depolarization by Cl^- channels. However, because the kinetics of depolarization were similar to the increases in intracellular calcium, it is proposed that opening of calcium activated chloride channels is the primary cause of the depolarization.

NRK cells become arrested in their growth at high cell densities (density-arrest) when cultured in the presence of EGF as the only growth factor present. Additional TGFβ, retinoic acid, or LPA causes a loss of this density-dependent growth arrest resulting in a phenotypic transformation of the cells. It has been shown that BK is able to block this growth stimulus-induced phenotypic transformation by a BK-specific receptor-mediated mechanism (Afink et al., 1994; van Zoelen et al., 1994). This effect of BK was not mimicked by $PGF_{2\alpha}$. Here, we show that $PGF_{2\alpha}$ transiently depolarizes quiescent NRK cells, as effectively as BK. This effect also was observed in density-arrested cells. Therefore, it is unlikely that the block of phenotypic transformation of NRK cells by BK is related to the currently observed depolarization of the cells.

Because confluent NRK fibroblasts are electrically well coupled (de Roos et al., 1996) and fibroblasts form cellular communicating networks in vivo (Komuro, 1989, 1990; Hasizume et al., 1992), agonist-induced changes in membrane potential may be implicated in intercellular signaling. Depolarizations evoked in a few cells may be transduced to unstimulated neighboring cells, thereby recruiting a greater population of cells to respond in unison to local stimuli. In other cells, membrane potential has been shown to be involved in the coordination of biological processes. For instance,

synchronous calcium oscillations in pancreatic islets (Santos et al., 1991) are mediated by changes in membrane potential. Also, it has been shown that electrical coupling between endothelial cells and smooth muscle cells is implicated in the transmission of a hyperpolarization (Bény and Paicicca, 1994), which may be involved in vasoconstriction.

We recently found that BK can generate propagating action potentials in NRK cells. The result of these action potentials was an almost synchronous increase in intracellular calcium in large numbers of cells (A.D.G. de Roos et al., submitted). These action potentials were generated by the regenerative opening of L-type calcium channels after an initial depolarization beyond a threshold value by BK. In the experiments described in this paper, a fast action potential-like depolarization occasionally preceded the BK-induced depolarization, for instance in low chloride media (Fig. 2B), which illustrates this phenomenon. In this way, signaling through depolarization by BK, $PGF_{2\alpha}$ and other phosphoinositide-mobilizing factors may be an effective way to propagate a signal between cells. We speculate that the depolarization evoked by calcium-mobilizing agonists and subsequent transmission to neighboring cells represents an important mechanism for fast intercellular transduction of locally evoked responses of cells to these agonists.

ACKNOWLEDGMENTS

We thank Peter Peters for technical assistance and Dr. Maarten Kansen for help with the combined patch-clamp and intracellular calcium measurements. This research was supported by the Netherlands foundation for life sciences (NWO-SLW).

LITERATURE CITED

Afink, G.B., Van Alewijk, D.C.G.J., de Roos, A.D.G., and van Zoelen, E.J.J. (1994) Lysophosphatidic acid and bradykinin have opposite effects on phenotypic transformation of normal rat kidney cells. J. Cell. Biochem., *56*:480–489.

Bény, J.-L., and Pacicca, C. (1994) Bidirectional electrical communication between smooth muscle and endothelial cells in the pig coronary artery. Am. J. Physiol., *266* (Heart Circ. Physiol. *35*):H1465–H1472.

Chao, A.C., Dix, J.A., Sellers, M.C., and Verkman, A.S. (1989) Fluorescence measurements of chloride transport in monolayer cultured cells. Mechanisms of chloride transport in fibroblasts. Biophys. J., *56*:1071–1081.

Clarke, L.L., Paradiso, A.M., Mason, S.J., and Boucher, R.C. (1992) Effects of bradykinin on Na^+ and Cl^- transport in human nasal epithelium. Am. J. Physiol., *262* (Cell Physiol. *31*):C644–655.

Cole, R.S., Fry, C.H., and Shuttleworth, K.E. (1988) The action of the prostaglandins on isolated human ureteric smooth muscle. Br. J. Urol., *61*:19–26.

Coutant, K.D., Corvaia, N., and Ryder, N.S. (1995) Bradykinin induces tyrosine phosphorylation of epidermal growth factor-receptor and focal adhesion proteins in human keratinocytes. Biochem. Biophys. Res. Commun., *210*:774–780.

de Roos, A.D.G., van Zoelen, E.J.J., and Theuvenet, A.P.R. (1996) Determination of gap junctional intercellular communication by capacitance measurements. Pfluegers Arch., *431*:556–563.

Fasolato, C., Pandiella, A., Meldolesi, J., and Pozzan, T. (1988) Generation of inositol phosphates, cytosolic Ca^{2+}, and ionic fluxes in PC12 cells treated with bradykinin. J. Biol. Chem., *263*:17350–17359.

Frace, A.M., and Gargus, J.J. (1989) Activation of single-channel currents in mouse fibroblasts by platelet-derived growth factor. Proc. Natl. Acad. Sci. USA, *86*:2511–2515.

Frizzell, R.A., and Morris, A.P. (1994) Chloride conductances of salt-secreting epithelial cells. Curr. Top. Membr., *42*:173–214.

Greger, R. (1990) Chloride channel blockers. Methods Enzymol., *191*:783–810.

Greger, R., and Kunzelmann, K. (1991) Simultaneous recording of the cell membrane potential and properties of the cell attached membrane of HT29 colon carcinoma and CF-PAC cells. Pfluegers Arch., *410*:487–494.

Hashizume, T., Imayama, S., and Hori, Y. (1992) Scanning electron microscopic study on dendritic cells and fibroblasts in connective tissue. J. Electron Microsc., *41*:434–437.

Higashida, H., and Brown, D.A. (1986) Two polyphosphatidylinositide metabolites control two K^+ currents in a neuronal cell. Nature, *323*:333–335.

Komuro, T. (1989) Three-dimensional observation of the fibroblast-like cells associated with the rat myenteric plexus, with special reference to the interstitial cells of Cajal. Cell Tissue Res., *255*:343–351.

Komuro, T. (1990) Re-evaluation of fibroblasts and fibroblast-like cells. Anat. Embryol., *182*:103–112.

Lahaye, D.H.T.P., Afink, G.B., Van Alewijk, D.C.G.J., and van Zoelen, E.J.J. (1994) Effect of bradykinin on loss of density-dependent growth inhibition of normal rat kidney cells. Cell. Mol. Biol., *40*:717–721.

Lamb, J.F., and McKinnon, M.G.A. (1971) The membrane potential and permeabilities of the L-cell membrane to Na, K, and chloride. J. Physiol., *213*:683–689.

Leeves, M.A., and McDonald, F. (1995) The effect of mechanical deformation on distribution of ions in fibroblasts. Am. J. Orthod. Dentofac. Orthop., *107*:625–632.

Lin, P., and Gruenstein, E. (1988) Pathways of chloride transport in human fibroblasts. Am. J. Physiol., *255*:C112–C122.

Mehrke, G., and Daut, J. (1990) The electrical response of cultured guinea-pig coronary endothelial cells to endothelium-dependent vasodilatators. J. Physiol., *430*:251–272.

Negishi, M., Sugimoto, Y., and Ichikawa, A. (1993) Prostanoid receptors and their biological actions. Prog. Lipid Res., *32*:417–434.

Pavenstädt, H., Bengen, F., Späth, M., Schollmeyer, P., and Greger, R. (1993) Effect of bradykinin and histamine on the membrane voltage, ion conductances and ion channels of human glomerular epithelial cells (hGEC) in culture. Pfluegers Arch., *424*:137–144.

Postma, F.R., Jalink, K., Hengeveld, T., Bot, A.G.M., Alblas, J., de Jonge, H.R., and Moolenaar, W.H. (1996) Serum-induced membrane depolarization in quiescent fibroblasts: Activation of a chloride conductance through the G protein-coupled LPA receptor. EMBO J., *15*:63–72.

Regoli, D., and Barabe, J. (1980) Pharmacology of bradykinin and related kinins. Pharmacol. Rev., *32*:1–46.

Roberts, R.A. (1989) *Bradykinin receptors: Characterization, distribution* and mechanisms of signal transduction. Prog. Growth Factor Res., *1*:237–252.

Rock, C.O., and Jackowski, S. (1987) Thrombin- and nucleotide-activated phosphatidylinositol 4,5-bisphosphate phospholipase C in human platelet membranes. J. Biol. Chem., *262*:5492–5498.

Santos, R.M., Rosario, L.M., Nadal, A., Garcia-Sancho, J., Soria, B., and Valdeolmillos, M. (1991) *Widespread synchronous* $[Ca^{2+}]_i$ oscillations due to bursting electrical activity in single pancreatic islets. Pfluegers Arch., *418*:417–422.

van Corven, E.J., Groenink, A., Jalink, K., Eichholtz, T., and Moolenaar, W.H. (1989) Lysophosphatidate-induced cell proliferation: Identification and dissection of signalling pathways mediated by G-proteins. Cell, *59*:45–54.

van den Eijnden-van Raaij, A.J.M., Koorneef, I., and van Zoelen, E.J.J. (1988) A new method for high yield purification of type beta transforming growth factor from human platelets. Biochem. Biophys. Res. Commun., *157*:16–23.

van Zoelen, E.J.J., Peters, P.H.J., Afink, G.B., van Genesen, S., de Roos, A.D.G., van Rotterdam, W., and Theuvenet, A.P.R. (1994) Bradykinin-induced growth inhibition of normal rat kidney cells is paralleled by a decrease in epidermal-growth-factor receptor expression. Biochem. J., *298*:335–340.

van Zoelen, E.J.J., Van Oostwaard, T.M.J., and de Laat, S.W. (1988) The role of polypeptide growth factors in phenotypic transformation of normal rat kidney cells. J. Biol. Chem., *263*:64–68.

Watanabe, T., Nakao, A., Emerling, D., Hashimoto, Y., Tsukamoto, K., Horie, Y., Kinoshita, M., and Kurokawa, K. (1994) Prostaglandin $F_{2\alpha}$ enhances tyrosine phosphorylation and DNA synthesis through phospholipase C-coupled receptor via Ca^{2+}-dependent intracellular pathway in NIH-3T3 cells. J. Biol. Chem., *269*:17619–17625.

White, M.M., and Aylwin, M. (1990) Niflumic and flufenamic acids are potent reversible blockers of Ca^{2+}-activated Cl^- channels in Xenopus oocytes. Mol. Pharmacol., *37*:720–724.