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Involvement of Protein Tyrosine Kinase in the InsP3-Induced Activation of Ca^{2+}-Dependent Cl^- Currents in Cultured Cells of the Rat Retinal Pigment Epithelium

O. Strauß[1], K. Steinhausen[1], S. Mergler[2], F. Stumpff[1], M. Wiederholt[1]

[1]Institut für Klinische Physiologie, Universitätsklinikum Benjamin Franklin, Freie Universität Berlin, Hindenburgdamm 30, 12200 Berlin, Germany

[2]Universitätsklinikum Charité der Humboldt-Universität zu Berlin, Medizinische Klinik, Hepatologie und Gastroenterologie, Campus Virchow Klinikum, Augustenburger Platz 1, 13353 Berlin, Germany

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Abstract. This combined study of patch-clamp and intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurement was undertaken in order to identify signaling pathways that lead to activation of Ca^{2+}-dependent Cl^- channels in cultured rat retinal pigment epithelial (RPE) cells. Intracellular application of InsP3 (10 μM) led to an increase in $[Ca^{2+}]_i$ and activation of Cl^- currents. In contrast, intracellular application of Ca^{2+} (10 μM) only induced transient activation of Cl^- currents. After full activation by InsP3, currents were insensitive to removal of extracellular Ca^{2+} and to the blocker of I_{CRAC}, La^{3+} (10 μM), despite the fact that both maneuvers led to a decline in $[Ca^{2+}]_i$. The InsP3-induced rise in Cl^- conductance could be prevented either by thapsigargin-induced (1 μM) depletion of intracellular Ca^{2+} stores or by removal of Ca^{2+} prior to the experiment. The effect of InsP3 could be mimicked by intracellular application of the Ca^{2+}-chelator BAPTA (10 mM). Block of PKC (chelerythrine, 1 μM) had no effect. Inhibition of Ca^{2+}/calmodulin kinase (KN-63, KN-92; 5 μM) reduced Cl^--conductance in 50% of the cells investigated without affecting $[Ca^{2+}]_i$. Inhibition of protein tyrosine kinase (50 μM tyrphostin 51, 5 μM genistein, 5 μM lavendustin) reduced an increase in $[Ca^{2+}]_i$ and Cl^- conductance. In summary, elevation of $[Ca]_i$ by InsP3 leads to activation of Cl^- channels involving cytosolic Ca^{2+} stores and Ca^{2+} influx from extracellular space. Tyrosine kinases are essential for the Ca^{2+}-independent maintenance of this conductance.

Key words: Retinal pigment epithelium — RPE — Chloride channels — Tyrosine kinase — Inositolphosphates

Correspondence to: O. Strauß

Introduction

The retinal pigment epithelium (RPE) is a monolayer of cells located between the choriocapillaris and the outer segments of photoreceptors. As part of the blood/retina barrier it is responsible for maintaining retinal function. The RPE transports ions and nutrients between blood and subretinal space, phagocytoses shed photoreceptor outer segments, secretes a variety of growth factors, and maintains the ion homeostasis of the subretinal space (Bok, 1985; Steinberg, 1985; Steele et al., 1992).

Cl^- conductances are involved in epithelial transport (Miller & Steinberg, 1977; Tsuboi & Pederson, 1988; Edelman & Miller, 1991; Joseph & Miller, 1991; LaCour, 1992; LaCour, 1993; Bialek & Miller, 1994; Hu et al., 1996), cellular volume regulation (Botchkin & Matthews, 1993; Kennedy, 1994), and in the compensation of changes in the subretinal ion composition (Griff, 1991; Fujii et al., 1992; Bialek, Joseph & Miller, 1993; Gallemore & Steinberg, 1993; LaCour, 1993). Activation mechanisms include induction by swelling (Botchkin & Matthews, 1993; Ueda & Steinberg, 1994), by phosphorylation (Strauß et al., 1998) and by elevation of intracellular Ca^{2+} (Ueda & Steinberg, 1994; Strauß, Wiederholt & Wienrich, 1996). Strauß et al. (1996) described the activation of Ca^{2+}-dependent Cl^- currents by intracellular application of inositol-1,4,5-triphosphate (InsP3). Ueda and Steinberg (1994) found that while swelling-induced Cl^- channels required intracellular Ca^{2+} for activation an additional unidentified cofactor is also necessary. Thus, application of a Ca^{2+}-ionophore is not a sufficient maneuver for activating Cl^- currents (Ueda & Steinberg, 1994), while elevation of cytosolic Ca^{2+} by backfilling into the patch pipette only leads to a transient effect (Strauß et al., 1996).

Table. Composition of bath solutions

Compound (mM)	Control Ringer	Low-Cl^- Ringer	Ca^{2+}-free Ringer	$NMDG^+$ Ringer
NaCl	135		137	
Na-methanesulfonate		120		
NMDG-Cl				135
$CaCl_2$	1	5		1
Ca-gluconate		5		
$MgCl_2$	0.6	0.6	0.6	0.6
HEPES	33	33	33	33
TEAC1	10	10	10	10
EGTA			1	
Glucose	6.1	6.1	6.1	6.1

Concentrations are given in mM. EGTA = ethylene glycol bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid, HEPES = N-(2-Hydroxyethyl)piperazine-N′-2-ethanesulfonic acid, $NMDG^+$ = N-methyl-D-glucanime, TEA^+ = tetraethylammonium.

The purpose of this study is the characterization of the additional components that are required for sustained activation of Ca^{2+}-dependent Cl^- channels in rat retinal pigment epithelial cells.

Materials and Methods

Cell Culture

Primary cultures of rat retinal pigment epithelial cells were established according to the method of Edwards (1977). After enucleation, eyes were stored overnight in Puck's saline F (Puck, Cieciura & Robinson, 1958). The eyes were incubated in Puck's saline F without Ca^{2+} and Mg^{2+} containing 0.1% trypsin for 35 min at 37°C. With a circumferential incision along the ora serata, the bulbi were opened and the anterior parts of the eyes including vitreous and retina were removed. Using a fine pair of forceps, the RPE was gently brushed away from Bruch's membrane and collected in Ham's F10 culture medium (supplemented with 20% fetal calf serum, 100 $\mu g ml^{-1}$ kanamycin and 50 $\mu g ml^{-1}$ gentamycin). After suspension of the RPE cells by gentle pipetting, the cell suspension was plated out into petri-dishes which were equipped with glass coverslips. The cultures were maintained at 37°C and 5% CO_2 in air; the medium was changed twice a week. After 24 hr, the cells had settled down and started to spread out. At the age of 3–8 days cultures were used for electrophysiological recordings.

Patch-Clamp Recordings

Patch-clamp experiments were performed at room temperature. Coverslips with RPE cells were placed into a perfusion chamber that was mounted onto the stage of an inverted microscope. The recordings were performed using K^+-free conditions to suppress superimposed potassium currents. The cells were superfused with the solutions indicated in the Table. Substances which were dissolved in DMSO containing stock solutions were added to bath solutions so that the final concentration of DMSO did not exceed 0.1%. This DMSO concentration has been shown not to influence the membrane conductance (Strauß et al., 1998). Patch pipettes of a resistance of 3–5 MΩ (in $NMDG^+$-containing solutions 9–11 MΩ) were pulled from borosilicate tubes using a Zeitz DMZ Universal puller (Zeitz; Augsburg, Germany). The standard pipette solution contained (mM): (i): 110 Cs-methanesulfonate, 20 NaCl, 2 $MgCl_2$, 10 HEPES, 0.1 EGTA, 0.1 fura-2-pentapotassium salt, (ii) BAPTA pipette solution: 100 Cs-methanesulfonate, 20 NaCl, 2 $MgCl_2$, 10 HEPES, 10 BAPTA, (iii) 10^{-5} M Ca^{2+}-pipette solution: 110 Cs-methanesulfonate, 20 NaCl, 2 $MgCl_2$, 10 HEPES, 0.01 $CaCl_2$, (iv) $NMDG^+$-containing pipette solution: 100 NMDG-methanesulfonate, 20 NMDG-Cl, 2 $MgCl_2$, 10 HEPES, 5.5 EGTA, 0.5 $CaCl_2$ (BAPTA = 1,2-bis(2-Aminophenoxy) Ethane-N,N,N′,N′-Tetraacetic acid). All solutions were adjusted to pH = 7.2 using Tris. InsP3 was added freshly from stock solutions to a final concentration of 10 μM. To avoid hyperosmotic swelling of the cells, the pipette solution was hypo-osmotic to the bath solution (app. 60 mOsm; measured using a Vapor Pressure Osmometer 5100B; Wescor, Logan, UT). Using these solutions, no changes in cell size could be observed during experiments in whole-cell configuration. The Nernst potential for Cl^- was with one of the pipette solutions and control Ringer −45 mV and with one of the pipette solutions and the low Cl^--Ringer +0.2 mV. Whole-cell currents were measured using an EPC-9 patch-clamp amplifier (HEKA, Lamprecht, Germany) and low-pass filtered at 3 kHz. Electrical stimulation, data storage and analysis were performed using the TIDA hard- and software (HEKA, Lamprecht, Germany) in conjunction with an AT-compatible computer. In experiments using pipette solutions with low Cl^- concentration (standard pipette solution, 10^{-5}M Ca^{2+} pipette solution and BAPTA pipette solution) with high Cl^- bath solutions (control Ringer and Ca^{2+}-free Ringer), all membrane potential values were corrected for the liquid junction potential of +10.7 mV (measured according to Neher (1992)). The membrane capacitance was calculated from the integration of the area below a transient capacitative currents curve which was induced by voltage-step of +10 mV for 30 msec. The access resistance was calculated from the relaxation time constant of the same capacitative currents, where the ratio of the time constant and the membrane capacitance yield the access resistance. The average resting potential of RPE cells was −39 ± 2 mV (n = 32), the membrane capacitance 40.9 ± 6.5 pF (n = 44) and the access resistance was 12.5 ± 1.2 MΩ (n = 45). Both membrane capacitance and access resistance were compensated before the actual measurement. Maximal currents amplitudes were measured from currents induced by a voltage-step from 0 mV to +90 mV for 50 msec.

To induce voltage-dependent currents, two major stimulation protocols were used. The step protocol consisted of nine voltage-steps of 10 mV increasing amplitude and 50 msec duration to depolarize the cell from a holding potential of 0 mV. This was followed by nine voltage-steps of 10 mV increasing amplitude and 50 msec duration to hyperpolarize the cell. The ramplike stimulation protocol was used to continuously monitor membrane conductance. For this purpose, membrane currents were continuously recorded at a holding potential of 0 mV and the cell was stimulated every 0.5 sec with a ramplike stimulation between −100 and +100 mV.

Measurements of Intracellular Ca^{2+}

For measuring intracellular Ca^{2+} ($[Ca^{2+}]_i$) in conjunction with whole-cell recordings of membrane conductance, a dual wavelength photometer (Luigs and Neumann, Ratingen, Germany) was connected to the same microscope used for patch-clamp experiments. Fluorescence of fura-2 was measured using the excitation wavelengths 360 and 390 nm at an emission wavelength of 510 nm. Fluorescence signals were digitalized by the built-in AD/DA converter of the EPC-9 patch-clamp amplifier so that fluorescence experiments could be fully monitored using TIDA hardware and software (HEKA, Lamprecht, Germany). Changes in intracellular free Ca^{2+} are presented as a ratio of the elicited

fluorescence at 360 and 390 nm. Before each measurement the autofluorescence and the fluorescence derived from the visible part of the patch-pipette were compensated.

The microscope was adjusted so that the fluorescence from only one cell was registered. To allow continuous monitoring of cytosolic Ca^{2+} concentration throughout the experiment, cells were preincubated with fura-2AM. For this purpose, RPE cells were incubated with fura-2AM (5 μM) dissolved in Ham's F10 culture medium without any supplements for 20 min in the dark. Subsequently, the cells were superfused for 20 min to remove residual fura-2AM. In addition, fura-2 (0.1 mM) was added to the pipette solution to minimize washout during the experiment.

Data Presentation

The figures usually show one experiment out of 3–6 performed. Data are presented as mean ± SEM. Statistical analysis was performed using student's *t*-test. Significance was considered at *P* values lower than 0.05.

Media and Substances

Media and supplements for cell culture were purchased from GIBCO-Life Technologies (Eggenstein, Germany) and Seromed (Berlin, Germany). The other substances were obtained from Sigma (Deisenhofen, Germany), Serva (Heidelberg, Germany), E. Merck (Darmstadt, Germany) and from RBI Research Biochemicals (Köln, Germany).

Results

Activation of Cl^- Currents by Intracellular Application of InsP3

In a first step, membrane conductance was measured using the perforated patch configuration (Horn & Marty, 1988). In the absence of potassium, at low extracellular Ca^{2+} concentration (using standard pipette solution with 150 μg/ml nystatin and control Ringer as bath solution) resting currents displayed negligible voltage-dependence. Current density in response to a voltage-step from 0 to +90 mV remained stable (value directly after establishing perforated-patch recording 1.02 ± 0.2 $pApF^{-1}$, value after 10 min 1.09 ± 0.25 $pApF^{-1}$; $n = 8$). The same could be observed in the whole-cell configuration without InsP3 in the pipette solution (current density directly after establishing the whole-cell configuration 0.83 ± 0.18 $pApF^{-1}$, after 10 min 1.3 ± 0.21 $pApF^{-1}$; $n = 8$).

In contrast, stimulation of RPE cells by intracellular application of inositol-1,4,5-trisphosphate (InsP3) led to a marked activation of voltage-dependent currents (Fig. 1). In these experiments, the cells were superfused with control Ringer solution and InsP3 (10 μM) was added to the standard pipette solution. Directly after breaking into the whole-cell configuration, the cells showed a resting potential of −39 ± 2 mV ($n = 32$) and no voltage-dependent currents could be observed. As InsP3 diffused into the cells, membrane conductance increased (Fig. 1*B*) from 0.98 ± 0.22 $pApF^{-1}$ ($n = 13$) to 128 ± 44 $pApF^{-1}$ ($n = 9$). Simultaneous monitoring of $[Ca^{2+}]_i$ (Fig. 1*C*) showed an increase of 198 ± 19% ($n = 5$) from a base level of 116 ± 32 ($n = 13$).

InsP3-induced currents showed an outwardly rectifying character with a reversal potential of −36.7 ± 1.3 mV ($n = 15$; Fig. 1*C* and *D*). These currents could be reduced to 12 ± 5% ($n = 5$) by application of 1 mM DIDS (4,4′-diisothiocyanatostilbene-2,2′ disulfonic acid) with recovery to 70 ± 9% ($n = 5$). InsP3 induced similar voltage-dependent currents when all cations were replaced by $NMDG^+$. In these experiments, a voltage step of only 20 mV (from a holding potential of 0 mV) was applied to exclude voltage errors as a result of the higher resistance of $NMDG^+$-containing solutions. Comparison of current density in the presence (3.7 ± 0.7 $pApF^{-1}$, $n = 7$) and absence (2.95 ± 0.2 $pApF^{-1}$, $n = 7$) of cations did not reveal significant differences.

Involvement of Cytosolic Ca^{2+} Stores

To clarify if InsP3 acts directly on Cl^- channels or if InsP3 acts via release of Ca^{2+} from cytosolic Ca^{2+} stores, cytosolic Ca^{2+} stores were depleted by thapsigargin before application of InsP3. In a first experiment, we studied the impact of thapsigargin (1 μM) itself on membrane conductance. Surprisingly, when thapsigargin was applied to cells in the perforated-patch configuration no effect on Cl^- conductance could be observed (Fig. 2). In a second experiment, thapsigargin (1 μM) was applied prior to InsP3 leading to a rise in $[Ca^{2+}]_i$ to 164.14 ± 18% ($n = 5$) of the base value (Fig. 3*A*). When $[Ca^{2+}]_i$ reached maximal levels, the whole-cell configuration was established (Fig. 3*B*) using the standard pipette solution with InsP3 (10 μM). Initially, no changes in $[Ca^{2+}]_i$ or membrane conductance occurred. Then, $[Ca^{2+}]_i$ began to decline, reaching after 5 min a level of 140 ± 12% ($n = 4$) of the basal level before application of thapsigargin. Conductance levels remained stable; in particular, no voltage-dependent currents could be detected (Fig. 3*C*).

Cl^- currents could also be activated by intracellular application of the Ca^{2+}-chelator BAPTA via the patch-pipette (Fig. 4*A*). Ninety seconds after establishing the whole-cell configuration with 10 mM BAPTA in the patch pipette the membrane conductance started to increase and reached maximal current amplitudes of 11.62 ± 2.7 $pApF^{-1}$ ($n = 5$, measured from voltage-step 0 to +90 mV) after 5 min in the whole-cell configuration. These currents showed a reversal potential of −34.4 ± 1.1 mV ($n = 5$).

Role of Extracellular Ca^{2+}

In a previous publication, we were able to demonstrate that intracellularly applied InsP3 failed to activate Cl^-

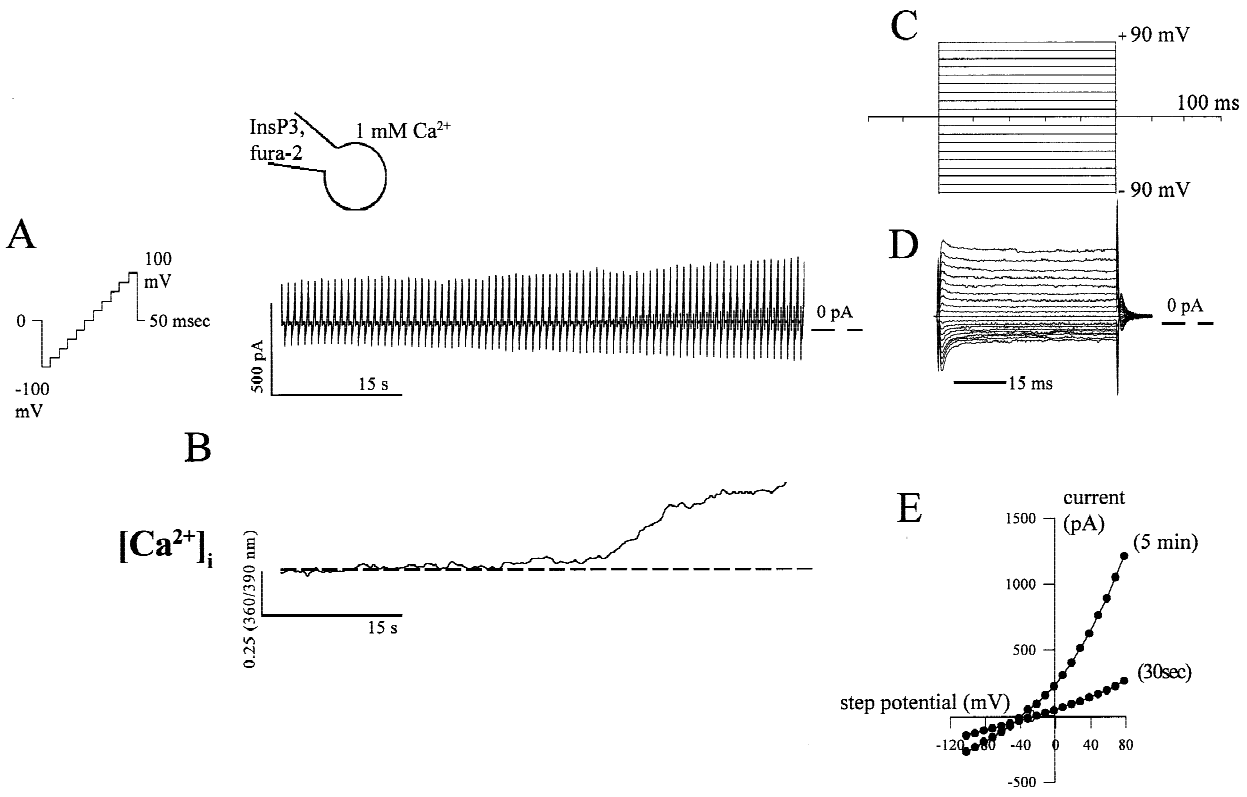

Fig. 1. Activation of Ca^{+}-dependent Cl^{-} channels by intracellular application of InsP3. The bath solution contained 1 mM Ca^{2+} (Control Ringer), the pipette solution 10 μM inositol-1,4,5-trisphophate and 100 μM fura-2. The cells were preincubated with fura-2AM. (*A*) Ramplike stimulation protocol which was used to monitor changes in membrane conductance. (*B*) Patch-clamp recording using the stimulation protocol in Fig. 1*A* which was started 60 sec after breaking into the whole-cell configuration. As InsP3 diffuses into the cell, membrane conductance started to increase. (*C*) Simultaneous recording of $[Ca^{2+}]_i$ measured by the fluorescence ratio of the two excitation wavelengths (360 nm : 390 nm). The rise in $[Ca^{2+}]_i$ is clearly associated with a concomitant rise in membrane conductance. (*D*) Step protocol used for measurement of currents depicted in Fig. 1*E*. (*E*) Current recording 5 min after breaking into the whole-cell configuration when membrane conductance reached maximal values (membrane capacitance 6 pF; dotted line indicates the zero-current level). (*F*) Current/voltage plot where maximal current amplitudes after 30 sec and 5 min in the whole-cell configuration with InsP3 in the pipette were plotted against their voltage-steps of the step-protocol to summarize an experiment with another cell (membrane capacitance 14 pF).

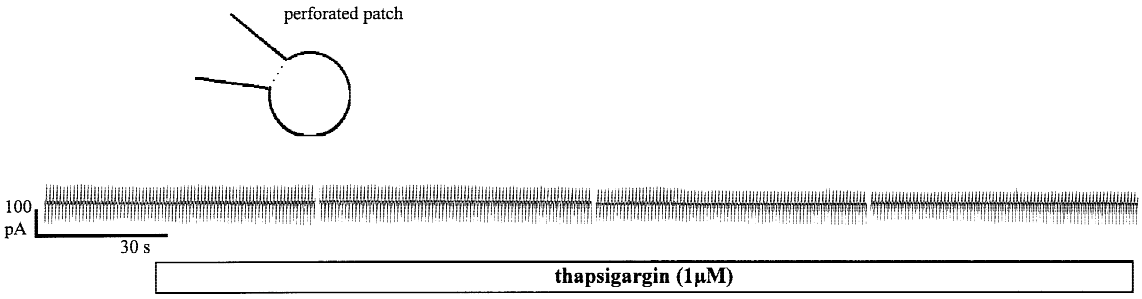

Fig. 2. Effect of thapsigargin during perforated-patch recording. 25 sec after establishing the perforated-patch recording (standard pipette solution supplemented with 150 μ/ml nystatin and control Ringer as bath solution) 1 μM thapsigargin was applied extracellularly. No change in membrane conductance could be observed during application of thapsigargin (membrane capacitance 12 pF; dotted line indicates zero current level).

currents under extra- and intracellular Ca^{2+}-free conditions (*see also* Fig. 4*B*), indicating that for the activation of Cl^{-} channels influx of extracellular Ca^{2+} is required. When Ca^{2+} was omitted from the extracellular solution, intracellular application of InsP3 led to maximal current amplitudes of 1.38 ± 0.39 $pApF^{-1}$ (n = 4, voltage-step from 0 to +90 mV), a value not significantly different from the resting membrane conductance. To mimic the

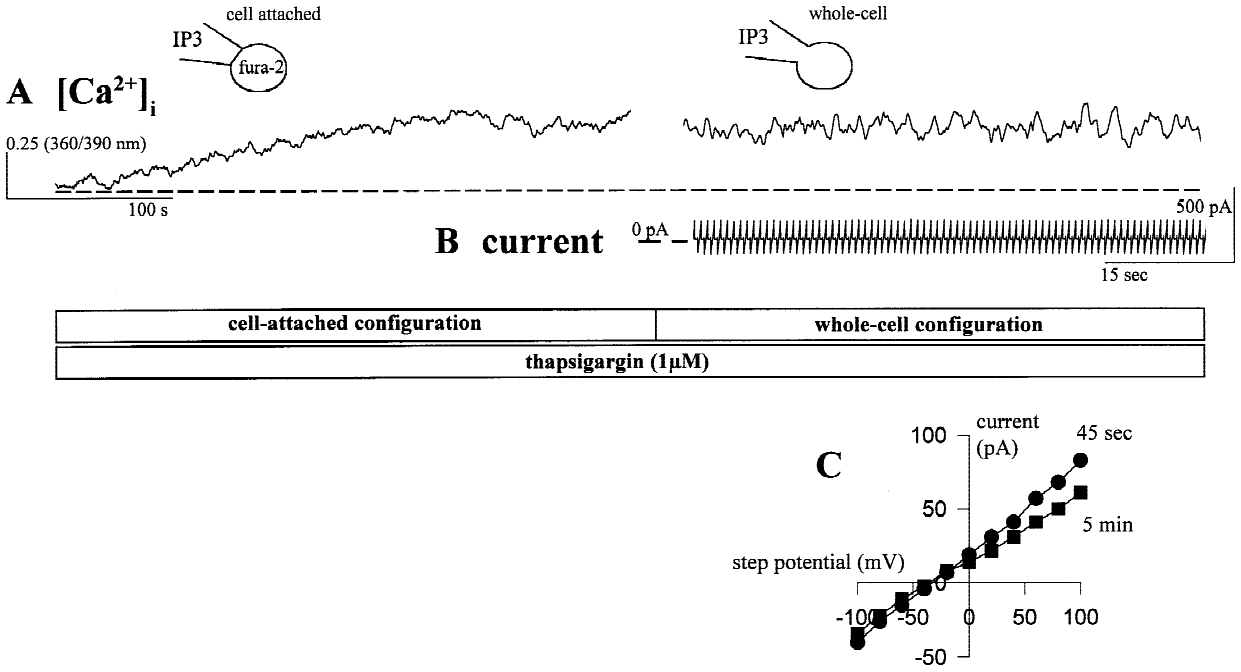

Fig. 3. Effect of preincubation of cells in thapsigargin. (*A*) Change in the intracellular Ca^{2+} depicted as fluorescence ratio before and after breaking into the whole-cell configuration with InsP3 in the patch pipette (Control Ringer as bath solution). The bar indicates the time of application of thapsigargin (1 μM). (*B*) Continuous recording of the membrane conductance 45 sec after breaking into the whole-cell configuration using the ramplike stimulation in Fig. 1*A* (membrane capacitance 50 pF; dotted line indicates the zero-current level). (*C*) Current/voltage plot of the membrane currents 45 sec after breaking into the whole-cell configuration and 5 min later (currents were plotted against the potentials of the ramplike stimulation).

effect of an influx of extracellular Ca^{2+} into the cell, we applied Ca^{2+} (10 μM) via the patch pipette. This maneuver led to transient activation of voltage-dependent currents which reached maximal current amplitudes of 25 ± 3.8 $pApF^{-1}$ ($n = 7$, voltage-step from 0 to +90 mV) after 60 sec in the whole-cell configuration. The currents showed a reversal potential of −37.2 ± 5.2 mV ($n = 5$) with asymmetrical intra- and extracellular Cl^--concentrations (using control Ringer in the bath and 10^{-5}M Ca^{2+} pipette solution) and a reversal potential of 0 mV with symmetrical Cl^- concentrations (low Cl^--Ringer and 10^{-5}M Ca^{2+} pipette solution) at the moment of maximal current activation. The membrane conductance returned to the resting level 15 sec later. With 100 μM Ca^{2+} in the patch pipette, stimulation of currents was so rapid that its rise escaped observation and we were only able to observe the return to the resting level.

To obtain further information on the role of Ca^{2+} for the activation of Cl^- channels, the effect of removing extracellular Ca^{2+} after activation of Cl^- channels was investigated (Fig. 5). For this purpose, the whole-cell configuration was established again with 10 μM InsP3 and fura-2 in the patch-pipette. As in previous experiments, this led to an increase in cytosolic free Ca^{2+} (Fig. 5*A*) and a subsequent activation of voltage-dependent Cl^- currents (Fig. 5*B*). Removal of extracellular Ca^{2+} after full activation of cytosolic free Ca^{2+} and membrane currents led to a decrease in intracellular Ca^{2+} to levels lower than the base levels observed before establishing the whole-cell configuration. After removal of extracellular Ca^{2+}, the cytosolic free Ca^{2+} was −33.5 ± 19% of the InsP3-induced Ca^{2+} level ($n = 7$; the negative value indicates a Ca^{2+} level below the resting level). Readdition of extracellular Ca^{2+} led to an intracellular Ca^{2+} level of 128 ± 13% of the InsP3-induced Ca^{2+} level ($n = 7$). In contrast, membrane conductance was not affected by both maneuvers.

Role of Protein Kinases

In the first set of experiments, the effect of a blocker for protein kinase C, chelerythrine, (Herbert et al., 1990) was studied. Extracellular application of chelerythrine (10 μM) did not influence InsP3-induced currents and cytosolic free Ca^{2+}. In the presence of chelerythrine, removal and readdition of extracellular Ca^{2+} decreased or increased the InsP3-induced Ca^{2+} level as in control conditions. In the presence of the Ca^{2+}/calmodulin kinase inhibitor KN-93 (5 μM) changes in intracellular Ca^{2+} induced by InsP3 or changes in extracellular Ca^{2+} after InsP3 application were not affected. However, in 50%

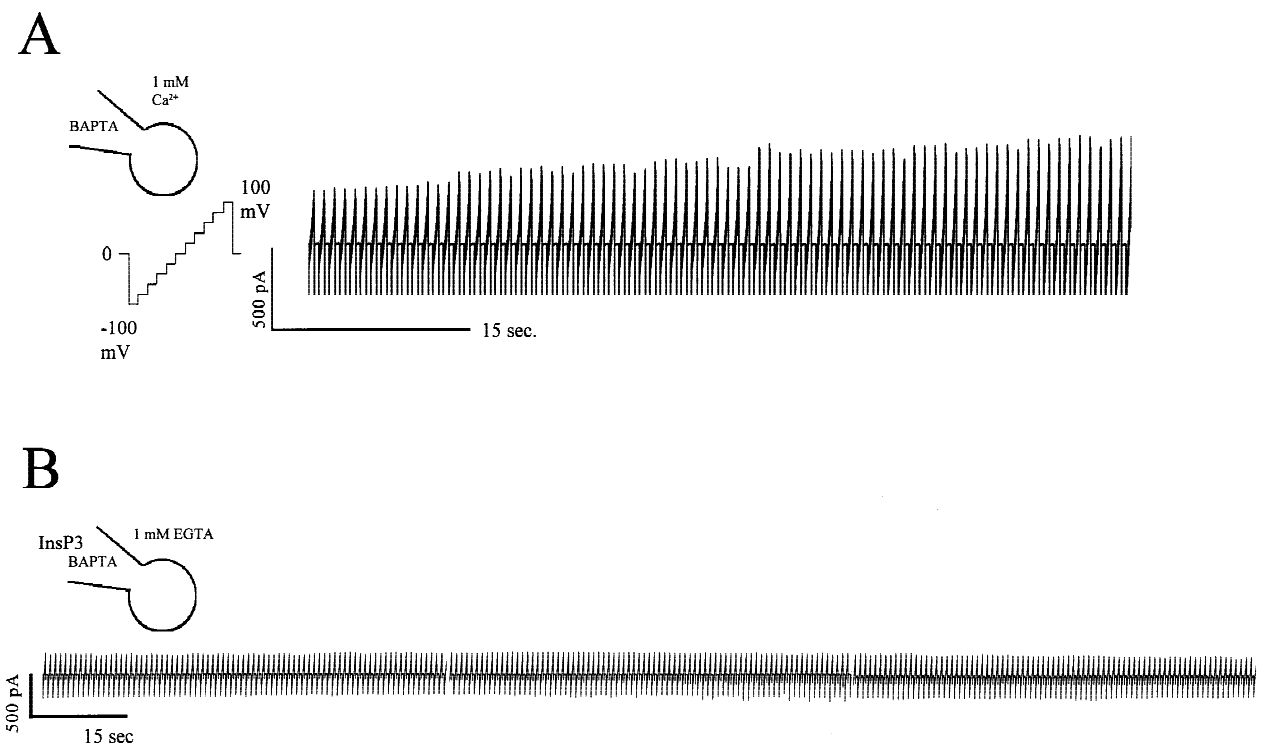

Fig. 4. Effect of removing Ca^{2+} on the activation of membrane conductance. (*A*) Effect of the intracellular application of the Ca^{2+} chelator BAPTA. The recording was started 30 sec after breaking into the whole-cell configuration. Intracellular application of BAPTA led to an increase in membrane conductance (membrane capacitance 35 pF, dotted line represents zero-current level). (*B*) Effect of intracellular application of InsP3 (10 μM) under intra- and extracellular Ca^{2+}-free conditions (using the BAPTA pipette solution and Ca^{2+}-free Ringer as bath solution). The recording was started 30 sec after breaking into the whole-cell configuration. Under these conditions, InsP3 failed to activate membrane currents (membrane capacitance 21 pF, dotted line indicates zero current level).

of the cells investigated, application of KN-93 after removal of extracellular Ca^{2+} led to a decrease in Cl^--current amplitude (Fig. 6*A*). This decrease was independent of the intracellular Ca^{2+}-concentration. In the presence of KN-93, the maximal current amplitude decreased to 36 ± 10% of control (recovery occurred to 85 ± 15%; $n = 3$). Comparable results could be observed using another blocker for Ca^{2+}/calmodulin kinase, KN-62 (5 μM). This substance did not influence cytosolic-free Ca^{2+}. In 3 of 6 cells, KN-62 (Fig. 6*A*) led to a reduction of maximal Cl^- current amplitude to 58 ± 13% ($n = 3$) with subsequent recovery to 89 ± 23% ($n = 3$).

In contrast to inhibition of serine/threonine kinases, the inhibition of protein tyrosine kinase had an effect on both the InsP3-induced Cl^--conductance and the InsP3-induced Ca^{2+} levels. For these experiments, Cl^- currents and cytosolic Ca^{2+} levels induced by intracellular application of InsP3 were fully activated. Application of a tyrosine kinase inhibitor, tyrphostin 51 (50 μM), reduced the Cl^- currents (Fig. 6*B*). Tyrphostin 51 reduced InsP3-induced currents to 63.4 ± 4% of control ($n = 6$); recovery occurred to 97.6 ± 3% of control ($n = 6$). The structurally different compound lavendustin was also able to reduce the current amplitudes of Ca^{2+}-dependent Cl^- channels (Fig. 6*B*). Extracellular application of lavendustin (1 μM) led to a reduction of InsP3-induced currents to 30 ± 11% of control (recovery occurred to 88 ± 9%; $n = 6$). The BAPTA-induced currents could also be reduced by tyrosine kinase inhibition. Application of tyrphostin 51 (50 μM) led to a reduction of BAPTA-induced currents to 63.2 ± 5.2% of control (recovery occurred to 118.8 ± 2.3%; $n = 3$). Readdition of extracellular Ca^{2+} in the presence of the tyrosine kinase blocker tyrphostin 51 did not increase intracellular Ca^{2+}, however, full recovery occurred after washout of tyrphostin 51 (Fig. 7*A–C*). A structurally different compound also influenced the InsP3-induced increase in cytosolic free Ca^{2+}. Genistein (5 μM) reduced the InsP3-induced increase in intracellular free Ca^{2+} to −17.9 ± 6.7% of control ($n = 3$; negative value indicates reduction below base line level); recovery occurred to 69 ± 6% of control ($n = 3$).

The Influx Pathway for Extracellular Ca^{2+}

La^{3+} is known to block I_{CRAC} Ca^{2+} channels (calcium release activated current) which are activated by deple-

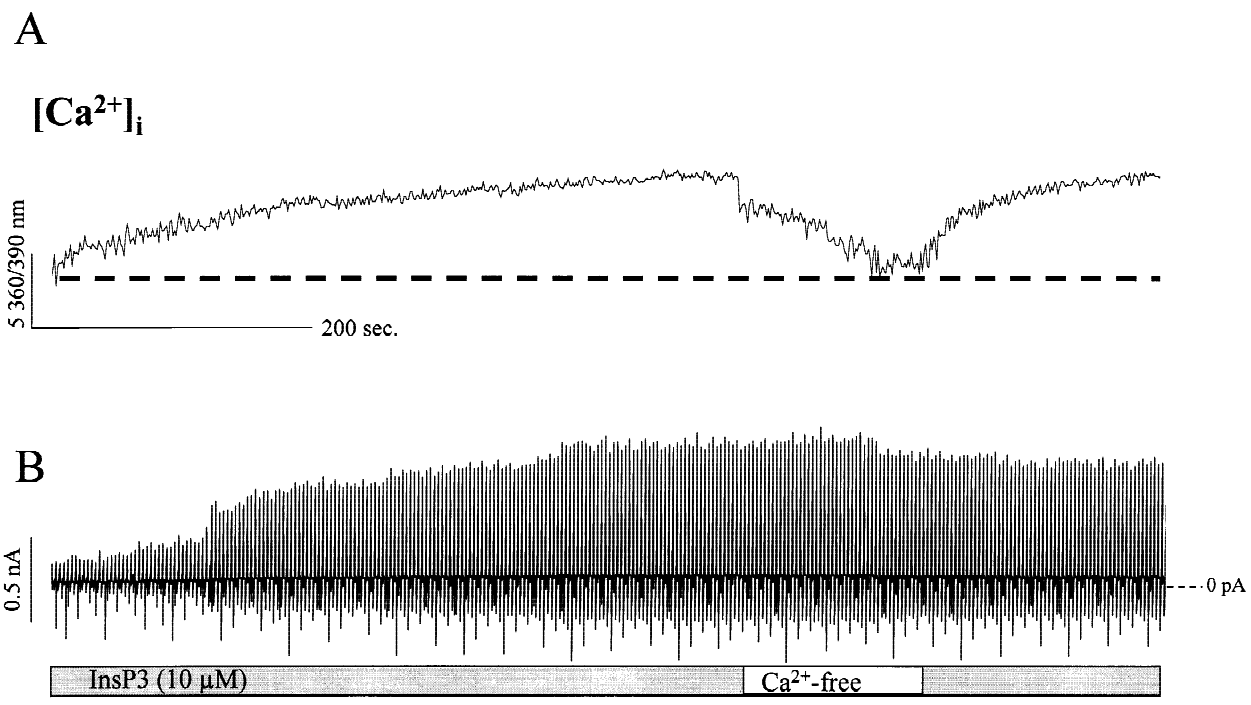

Fig. 5. Effect of removing extracellular Ca^{2+} after full activation of InsP3-induced currents. (*A*) Change in intracellular free Ca^{2+} depicted as the ratio of the two excitation wavelengths after breaking into the whole-cell configuration with InsP3 in the pipette. The bar indicates the time of removal of extracellular Ca^{2+} (using Ca^{2+}-free Ringer as bath solution). (*B*) Simultaneous registration of membrane currents. Removing extracellular Ca^{2+} decreased intracellular Ca^{2+} to base line level but did not alter InsP3-induced membrane conductance. The decrease after readding extracellular Ca^{2+} is due to rundown during the whole-cell configuration (membrane capacitance 21 pF; dotted line indicates zero current level).

tion of InsP3-sensitive cytosolic Ca^{2+} stores (Hoth & Penner, 1993). Extracellular application of La^{3+} (10 μM, resulting in 90% inhibition) reversibly reduced the InsP3-induced level of intracellular free Ca^{2+} (Fig. 8*A* and *C*). The membrane conductance remained unchanged in the presence of La^{3+} (Fig. 8*B* and *D*). In a final series of experiments, the blocker of nonselective cation channels, flufenamic acid (1 μM), was applied to the cells after maximal stimulation by InsP3. This maneuver had no effect, either on levels of $[Ca^{2+}]_i$ or on membrane conductance.

Discussion

In this study we were able to show that intracellularly applied inositol-1,4,5-triphosphate (InsP3) to cultured rat retinal pigment epithelial cells releases Ca^{2+} from cytosolic stores. Subsequently, activation of tyrosine kinase leads to activation of an influx of extracellular Ca^{2+} into the cell. This increase in cytosolic-free Ca^{2+} leads to an activation of voltage-dependent Cl^- channels. For a sustained activation of these Cl^- channels, the activity of tyrosine kinase was required.

Since we used a hypotonic pipette solution (difference of 60 mOsm), it is unlikely that swelling induced currents mimic an InsP3 effect. The reduced ionic strength of the intracellular solution cannot be an activator of Cl^- currents as has been reported by Nilius et al. (1998). A reduced ionic strength only activates Cl^- channels when intra- and extracellular solutions are isoosmotic. A switch to a hyperosmotic bath solution reverses the effects of a reduced intracellular ionic strength. In addition, the activation of Cl^- channels in RPE cells is clearly dependent on the presence of InsP3 in the pipette solution. When using the same solutions in perforated-patch recordings with nystatin or without InsP3 in the patch-pipette in whole-cell recordings no changes in membrane conductance could be observed.

Mechanism of InsP3-Induced Increase in Cytosolic Free Ca^{2+}

Intracellular application of InsP3 to RPE cells led to an increase in free cytosolic Ca^{2+}. Possibly, this is enabled by a direct opening of Ca^{2+} channels by InsP3 (Kuno & Gardner, 1987; Kuno, Maeda & Mikoshiba, 1994) or by release of Ca^{2+} from InsP3-sensitive cytosolic Ca^{2+} stores (Marty & Tan, 1989; Matthews, Neher & Penner, 1989; Hoth & Penner, 1993). Extracellular application of thapsigargin, a substance which depletes cytosolic

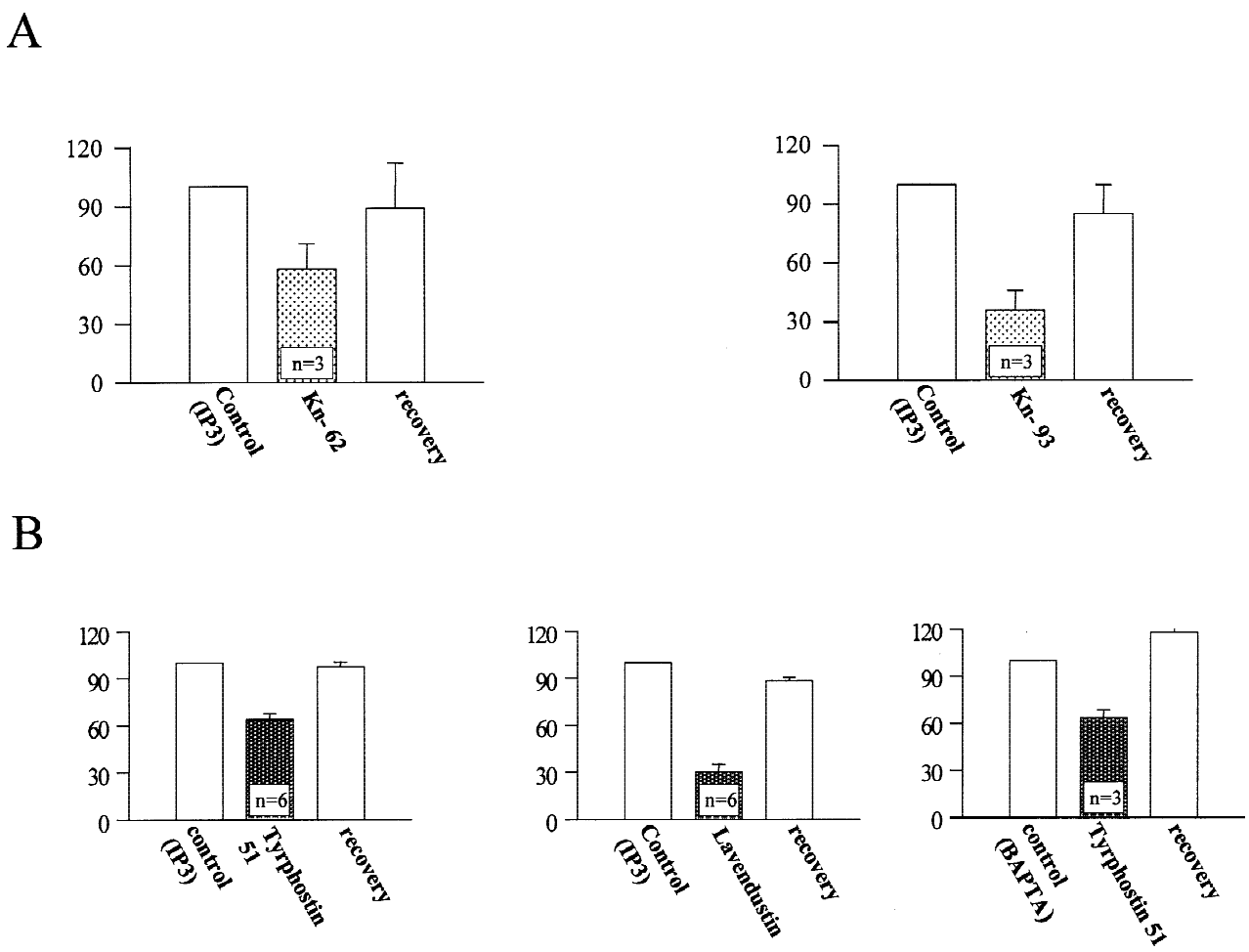

Fig. 6. Effect of the inhibition of protein kinase on Ca^{2+}-dependent Cl^- currents. (*A*) Effect of Ca^{2+}/calmodulin kinase inhibitors was observed in 50% of the investigated cells. Left panel: Effect of KN-62 (5 μM) on InsP3-induced currents ($n = 3$). Right panel: Effect of KN-93 (5 μM) on InsP3-induced currents ($n = 3$). (*B*) Effect of protein tyrosine kinase inhibitors. Maximal current amplitudes were measured using a voltage-step from 0 to +90 mV. Current amplitude before application of the blocker was set at 100%. Left panel: Effect of tyrphostin 51 (50 μM) on InsP3-induced currents ($n = 6$). Middle panel: Effect of lavendustin A (5 μM) on InsP3-induced currents ($n = 6$). Right panel: Effect of tyrphostin 51 (50 μM) on BAPTA induced currents ($n = 3$).

Ca^{2+} stores by inhibition of their Ca^{2+}-ATPase, led to an increase in cytosolic free Ca^{2+} in RPE cells. When InsP3 was applied intracellularly after this maneuver, no further changes in cytosolic free Ca^{2+} could be observed. Thus, InsP3 acts via release of Ca^{2+} from cytosolic Ca^{2+} stores. This is supported by the observation that intracellular application of BAPTA also led to an activation of Cl^- current. However, BAPTA was not as effective as InsP3 in activating Cl^- currents. To clarify the origin of the InsP3-induced increase in cytosolic free Ca^{2+}, extracellular Ca^{2+} was removed after the InsP3-induced Ca^{2+}-increase reached the maximal level. This maneuver led to a decrease of intracellular Ca^{2+} below the base level which fully recovered to the original level when extracellular Ca^{2+} was readded. Thus, InsP3 seems to increase cytosolic free Ca^{2+} by store-depletion-dependent induction of a Ca^{2+} influx from the extracellular space. Ca^{2+} influx into the cell could be reduced by extracellular application of La^{3+}, but not by flufenamic acid. In the concentration we used, La^{3+} inhibits 90% of the currents through I_{CRAC} channels (Hoth & Penner, 1993). Flufenamic acid is a well known inhibitor of nonspecific cation channels. Both substances did not influence the InsP3-induced Cl^--conductance. In addition, the membrane potential was clamped to 0 mV, which inactivates L-type Ca^{2+} channels. Since the intracellular application of InsP3 induced large Cl^- currents, it was not possible to measure currents through I_{CRAC} channels directly. Developing methods to measure currents through I_{CRAC} channels in the presence of large Cl^- currents was beyond the scope of this paper.

Mechanisms Activating Cl^- Conductance

The InsP3-induced Ca^{2+} influx into the cell activates Cl^- channels. The identification of InsP3-induced conduc-

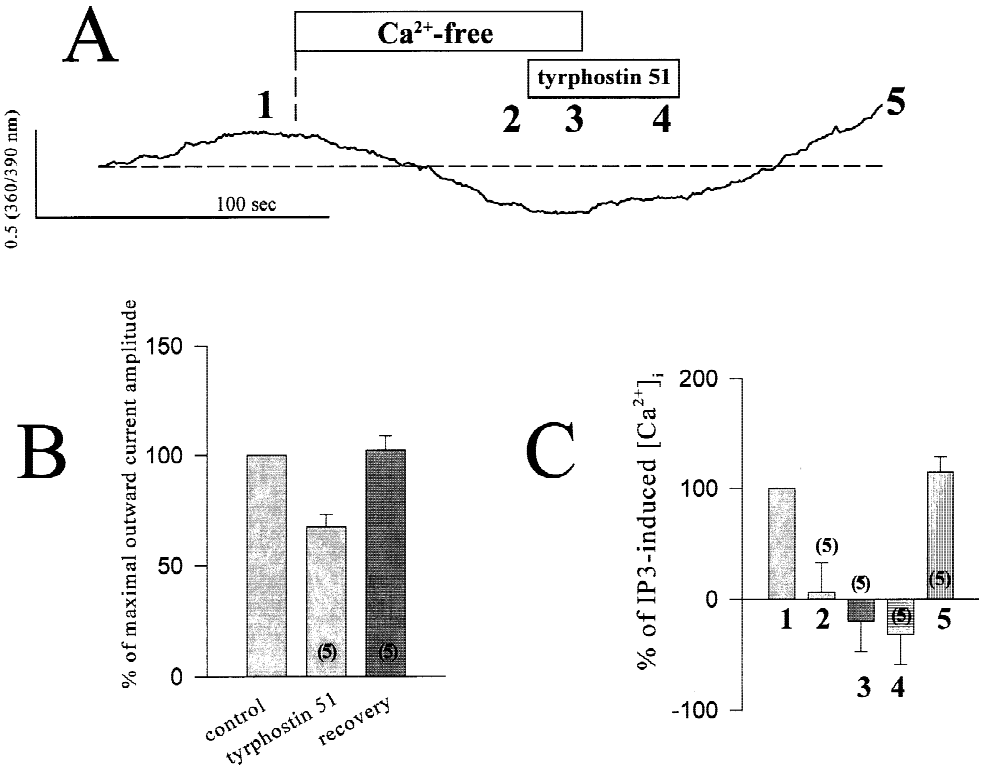

Fig. 7. Effect of tyrphostin 51 on InsP3-induced membrane conductance and rise in intracellular Ca^{2+}. (*A*) Change in intracellular free Ca^{2+} depicted as fluorescence ratio of the two excitation wavelengths 360 nm and 390 nm. The recording was started after breaking into the whole-cell configuration with InsP3 in the patch pipette (Control Ringer as bath solution). The rectangles indicate the duration during which extracellular Ca^{2+} was absent (Ca^{2+}-free Ringer as bath solution) and the presence of tyrphostin 51 (50 μM). (*B*) Changes in maximal outward current amplitude (induced by a voltage-step from 0 to +90 mV) induced by tyrphostin 51. Values are given in per cent of the control value (SEM; tyrphostin 51 and recovery were significantly different). (*C*) Changes in intracellular Ca^{2+} during the experiments. The numbers correspond to the numbers in 7*A* and indicate when intracellular Ca^{2+} was estimated. The levels in intracellular Ca^{2+} were expressed in per cent of the InsP3-induced rise in intracellular Ca^{2+}. Negative values indicate a reduction of the intracellular Ca^{2+} below the base level. In some cells, it appeared that when extracellular Ca^{2+} was readded in the presence of tyrphostin that intracellular free Ca^{2+} started to increase. However, the values of intracellular Ca^{2+} were not significantly different between extracellular Ca^{2+}-free conditions and the presence of tyrphostin. The values "2", "3" and "4" were not significantly different among each other but significantly different to "5".

tance has been reported in a previous publication (Strauß et al., 1996). Several reasons speak for chloride as the ion responsible for InsP3-induced currents. The currents are sensitive to the chloride channel blocker DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid). In addition, these currents could be induced by InsP3 when all cations were replaced by $NMDG^+$. While reversal potentials equal the Nernst potential for symmetrical Cl^--concentrations, the reversal potential of InsP3-induced currents is more positive than the Nernst potential for Cl^- under asymmetrical Cl^--concentrations. However, this is to be expected as the InsP3-induced Ca^{2+}-dependent Cl^--channel is able to conduct methanesulfonates which were used to replace Cl^-ions (Calahan & Lewis, 1988). The influx of extracellular Ca^{2+} into the cell activates Cl^- conductance. When Ca^{2+} was omitted from intra- and extracellular solutions, InsP3 failed to activate Cl^- currents (Strauß et al., 1996). Thus, InsP3 activates Ca^{2+}-dependent Cl^- channels. The Ca^{2+}-dependent Cl^- channels in RPE cells showed an outwardly rectifying character and a fast voltage-dependent activation which is unusual for other known Ca^{2+}-dependent Cl^- channels. In addition, the Ca^{2+}-dependent Cl^- channel in RPE cells can be blocked by DIDS and not by the fenamate compound flufenamic acid. With these characteristics the channels resemble the epithelial Ca^{2+}-dependent Cl^- channel cloned by Cunningham et al. (1995) which activates in the same manner and which can be blocked by DIDS and not by the fenamate compound niflumic acid. However, Ca^{2+} alone is not sufficient for a sustained activation of Cl^- channels. The intracellular application of Ca^{2+} only leads to a transient activation of Cl^- currents. In these experiments, it was difficult to find the right Ca^{2+} concentration in the pipette solution. 1 μM was too small and with 100 μM, only the return of membrane conductance back to the resting level could be observed. This is also the explanation for the observation that application of a Ca^{2+}-ionophore did not succeed in activation of Cl^- currents (*not shown*). Using this method, it is impossible to induce the right intracellular

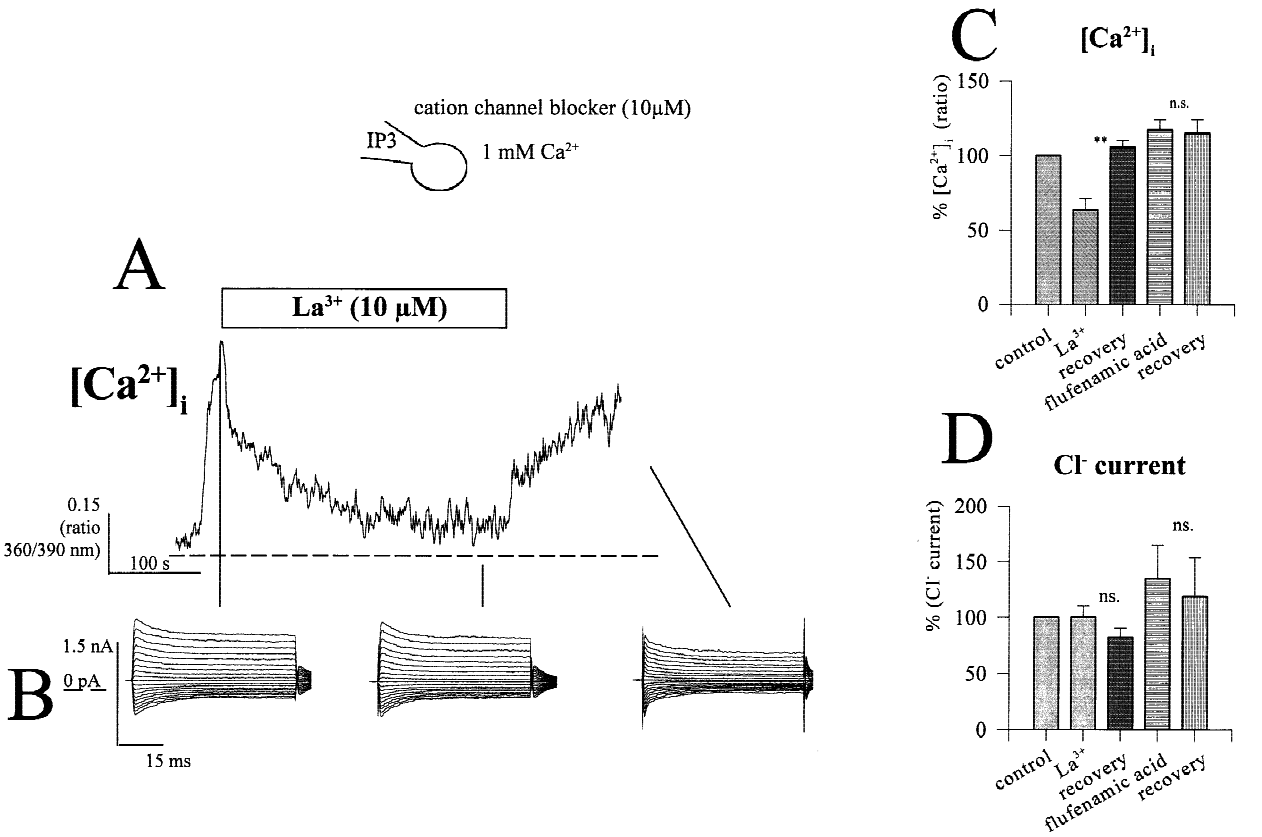

Fig. 8. Effect of blockers for Ca^{2+}-influx pathways on the InsP3-induced rise in intracellular Ca^{2+}. (*A*) Change in intracellular free Ca^{2+} depicted as fluorescence ratio of the two excitation wavelengths 360 nm and 390 nm after breaking into the whole-cell configuration with InsP3 in the patch pipette (Control Ringer bath solution). The rectangle indicates the time of La^{3+} (10 μM) application. (*B*) Voltage-dependent at the time of maximal InsP3-induced rise in intracellular Ca^{2+} (left panel), the presence of La^{3+} (middle panel) and recovery from La^{3+} (right panel; membrane capacitance 16 pF; dotted line indicates the zero-current level). (*C*) Summary ($n = 5$) of the effects of La^{3+} (10 μM) and flufenamic acid (1 μM) on intracellular free Ca^{2+}. The InsP3-induced Ca^{2+} level was set as 100%. (*D*) Summary ($n = 5$) of the effects La^{3+} (10 μM) and of flufenamic acid (1 μM) on the maximal outward current amplitude (induced by a voltage-step from 0 to +90 mV). The current amplitude before application of the blocker was set as 100%.

Ca^{2+} concentration to see this transient activation of Cl^- channels. Failure in inducing the right Ca^{2+} concentration may also be the explanation for the inability of thapsigargin to induce chloride currents. In addition, removing extracellular Ca^{2+} after full activation of Cl^--conductance and full rise of free cytosolic Ca^{2+} led to a reversible decrease of intracellular Ca^{2+} but did not change Cl^- currents. Thus, after full activation, Ca^{2+} is no longer required to maintain the Cl^- conductance activated by Ca^{2+}. An additional factor which is not Ca^{2+}-dependent and activated by release of Ca^{2+} from cytosolic stores is required for Cl^- channel activation. The activation of this factor seems to be dependent on the way in which Ca^{2+} is released from cytosolic stores. Using InsP3 or passive depletion of stores by intracellular application of the Ca^{2+}-chelator BAPTA, Ca^{2+}-dependent chloride currents could be activated. Using thapsigargin to deplete Ca^{2+} stores by inhibition of the Ca^{2+}-ATPase of these stores did not lead to activation of chloride currents.

Observations pointing in the same direction were made previously by Ueda and Steinberg (1994). These authors failed to activate Ca^{2+}-dependent Cl^- channels by extracellular application of ionomycin in the presence of extracellular Ca^{2+}. However, if the authors (Ueda & Steinberg, 1994) first incubated the cell in ionomycin under extracellularly Ca^{2+}-free conditions and readded extracellular Ca^{2+} in the presence of ionomycin, they were able to activate Ca^{2+}-dependent Cl^- channels in RPE cells. This maneuver resembles a maneuver used to induce capacitative Ca^{2+} entry. We suggest that the data from Ueda and Steinberg (1994) can be interpreted if one assumes that Ca^{2+}-dependent Cl^- channels need factors which are activated by the release of Ca^{2+} from cytosolic stores for a sustained activation. We propose that the right intracellular Ca^{2+} concentration in combination with a factor that is activated by depletion of cytosolic Ca^{2+} stores is required for sustained activation of Ca^{2+}-dependent chloride channels in RPE cells. The additional factor enables sustained activity of the chloride

channels independent from Ca^{2+}. In addition, this factor can only be activated by certain mechanisms of store depletion, of which InsP3 is the most efficient.

Factors Activated by Depletion of Ca^{2+} Stores

To characterize the factors that are activated by release of Ca^{2+} from intracellular stores, we investigated the effect of inhibiting various protein kinases. Inhibition of protein kinase C by chelerythrine (Herbert et al., 1990) neither changed the InsP3-induced rise in cytosolic free Ca^{2+} nor the activation of Cl^- currents. Ca^{2+}/calmodulin kinase blocker KN-62 (Tokumitsu et al., 1990) and KN-93 (Sumi et al., 1991) showed no effect on the pathway which stimulates calcium influx from extracellular space. However, in 50% of the investigated cells, these substances reduced the InsP3-induced Cl^- current. Since the effects of KN-93 and -62 on Cl^- currents were observed under extracellularly Ca^{2+}-free conditions, Ca^{2+} appears as a trigger to initialize kinase activity. Thus, the involvement of Ca^{2+}/calmodulin kinase in activating Ca^{2+}-dependent Cl^- channels cannot be excluded. Interestingly, data from other tissues on regulation of Ca^{2+}-dependent Cl^- channels by Ca^{2+}/calmodulin kinase are contradictory. In smooth muscle Ca^{2+}/calmodulin kinase led to inactivation of these channels (Wan & Kotlikoff, 1997). In epithelial cells, these ion channels (Fuller et al., 1994; Schlenker & Fitz, 1996) are activated.

In contrast to these serine/threonine kinases, protein tyrosine kinase (PTK) seemed to be involved in the communication between Ca^{2+} stores and the cell membrane. Extracellular application of tyrphostin 51 or lavendustin reversibly reduced the InsP3-induced Ca^{2+}-dependent Cl^- currents. In addition, inhibition of tyrosine kinase could also block Cl^- currents induced by intracellular application of BAPTA. Thus, protein tyrosine kinases are needed in addition to Ca^{2+} for activation of Cl^- channels. Tyrphostin 51 also prevented the recovery from the decrease in cytosolic free Ca^{2+} induced by removal of extracellular Ca^{2+} after Cl^- currents and intracellular Ca^{2+} were maximally stimulated by InsP3. In addition, genistein reduced the InsP3-induced $[Ca^{2+}]_i$ levels to levels lower than the resting level. Thus, tyrosine kinases are involved in the induction of the store depletion-induced influx of extracellular Ca^{2+} into the cell. The induction of a Ca^{2+} influx by release of Ca^{2+} from cytosolic stores via activation of tyrosine kinase has been observed by other groups (Low, 1996; Marhaba et al., 1996; Sharma & Davis, 1996).

Activation of Cl^- Channels by Tyrosine Kinase

The participation of tyrosine kinase in Cl^- channel regulation has become a topic of interest recently. In studies investigating activation mechanisms for the CFTR (cystic fibrosis transmembrane regulator) Cl^- channels, tyrosine kinases appeared as inhibitors of Cl^- channels. Inhibition of tyrosine kinase seems to activate CFTR Cl^- channels independently of the cAMP pathway (Illek et al., 1995; Sears et al., 1995; Shuba et al., 1996; Yang et al., 1997). However, the observations made in these studies are somewhat contradictory. French et al. (1997) showed that the most commonly used blocker of tyrosine kinases, genistein, is able to interact directly with CFTR channels. The effect of tyrosine kinase on CFTR channels is more likely to be enabled by inhibition of phosphatases which in turn activate CFTR channels (Yang et al., 1997). Tyrosine kinases appear as an activator of swelling induced Cl^- channels (Crépel et al., 1998; Lepple-Wienhues et al., 1998) or of Cl^- channels involved in the initiation of apoptosis (Szabo et al., 1998). Until now, the role of tyrosine kinases in activation of Ca^{2+}-dependent Cl^- channels was believed to be that of an activator of phospholipase C which initializes the Ca^{2+}/InsP3-second messenger system (Marrero et al., 1996). More directly, Ca^{2+}-dependent Cl^- channels have been shown to be inhibited by tyrosine phosphorylation via increase of their Ca^{2+}-sensitivity (Shintani & Marunaka, 1996). In our study, Ca^{2+} appears as the initiator of Cl^--conductance, whereas tyrosine kinase keeps Cl^- channels continuously activated independent of Ca^{2+}. However, using the patch-clamp technique it is not possible to demonstrate the direct interaction of tyrosine kinase and Cl^- channels as has been discussed for investigations concerning the direct interaction of G-proteins and ion channels (Clapham, 1994).

Ca^{2+}-Dependent Cl^- Channels and RPE Function

Chloride conductances have been found to be of functional importance for the RPE. They provide efflux pathways out of the cell for epithelial transport (Miller & Steinberg, 1977; Tsuboi & Pederson, 1988; Edelman & Miller, 1991; Joseph & Miller, 1991; LaCour, 1992; LaCour, 1993; Bialek & Miller, 1994; Hu et al., 1996), enable cellular volume regulation (Botchkin & Matthews, 1993; Kennedy, 1994) and maintain subretinal ion homeostasis during electrical activity of the photoreceptors (Griff, 1991; Fujii et al., 1992; Bialek et al., 1993; Gallemore & Steinberg, 1993; LaCour, 1993). In the present study we showed that release of Ca^{2+} from cytosolic InsP3-sensitive stores activates Cl^- channels. InsP3 is known to be stimulated by several agonists like neuropeptides (Kuriyama et al., 1992), vasopressin (Friedman et al., 1991) and carbachol (Liu et al., 1992). InsP3 triggers the onset of phagocytosis (Rodriguez de Turco, Gordon & Bazan, 1992). Light stimulates the generation of InsP3 (Rodriguez de Turco et al., 1992) and also stimulates basolateral chloride conductance

(Gallemore & Steinberg, 1993). We suggest that the light-induced increase in basolateral chloride conductance may be due to InsP3-induced activation of Ca^{2+}-dependent Cl^- channels. InsP3 itself may be induced by an agonist which diffuses from the retina to the cell after light stimulation (Gallemore & Steinberg, 1993).

In summary, we were able to show that a Ca^{2+}-dependent Cl^- conductance is activated subsequent to InsP3-dependent Ca^{2+} release from cytosolic stores. The conductance is initiated by activation of Ca^{2+} influx from the extracellular space. For chloride conductance to remain activated over a length of time, tyrosine kinase activity is essential. Possibly, tyrosine kinases are involved to provide the link between depleted Ca^{2+} stores and the subsequent induction of an influx of extracellular Ca^{2+}.

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