J. Membrane Biol. 187, 71–79 (2002)
DOI: 10.1007/s00232-001-0149-3

The Journal of
Membrane
Biology

© Springer-Verlag New York Inc. 2002

Regulation of a Calcium-sensitive K^+ Channel (cIK1) by Protein Kinase C

A. Wulf, A. Schwab
Physiologisches Institut, Universität Würzburg, Röntgenring 9, D-97070 Würzburg, Germany

Received: 22 August 2001/Revised: 15 January 2002

Abstract. Ca^{2+}-sensitive K^+ channels (IK1 channels) are required for many physiological functions such as cell proliferation, epithelial transport or cell migration. They are regulated by the intracellular Ca^{2+} concentration and by phosphorylation-dependent reactions. Here, we investigate by means of the patch-clamp technique mechanisms by which protein kinase C (PKC) regulates the canine isoform, cIK1, cloned from transformed renal epithelial (MDCK-F) cells. cIK1 elicits a K^+-selective, inwardly rectifying, and Ca^{2+}-dependent current when expressed in HEK293 or CHO cells. It is inhibited by charybdotoxin, clotrimazole, and activated by 1-ethyl-2-benzimidazolone. cIK1 is activated by intracellular application of ATP or ATP[γS]. ATP-dependent activation is reversed by PKC inhibitors (bisindolylmaleimide, calphostin C), while stimulation with ATP[γS] resists PKC inhibition. Stimulation of protein kinase C with phorbol 12-myristate 13-acetate (PMA) leads to the acute activation of cIK1 currents, which are blocked by PKC inhibitors. In contrast, PKC depletion by overnight incubation with PMA prevents ATP-dependent cIK1 activation. Neither single mutations nor the simultaneous mutation of all PKC sites (T101, S178, T329) to alanine alter the acute regulation of cIK1 channels by PKC. However, current amplitudes of CIK1-T329A and the triple mutant are dramatically increased upon long-term treatment with PMA. These mutations thereby disclose an inhibitory effect on cIKl current of the PKC site at T329. Our results indicate that cIK1 channel activity is regulated in two ways. PKC-dependent activation of cIK1 channels occurs indirectly, while the inhibitory effect probably requires a direct interaction with the channel protein.

Correspondence to: A. Schwab; email: albrecht.schwab@mail.uni.wuerzburg.de

Key words: Ca^{2+}-sensitive K^+ channels — Protein kinase C — Patch clamp

Introduction

Ca^{2+}-sensitive K^+ channels with intermediate conductance (IK1 channels) are expressed in many different cell types such as secretory epithelial cells [2, 31], endothelial cells [13], fibroblasts [21], T lymphocytes [6], melanoma cells [18], granulocytes [32], macrophages [23], erythrocytes [20], or cultured cell lines [7, 27]. Thus, IK1 channels are required for a wide variety of physiological functions ranging from transepithelial secretion to T cell activation. Interestingly, many of the nonepithelial cells expressing IK1 channels have the ability to migrate. Our studies showed that cell migration is also modulated by the activity of IK1 channels [26, 28–30].

The intracellular Ca^{2+} concentration is the most important regulator of IK1 channels. The Ca^{2+} sensitivity is conferred to the channel protein by the association of its C-terminus with calmodulin [5]. IK1 channels are further regulated by phosphorylation-dependent reactions. This is of physiological importance since IK1-channel activation frequently requires the stimulation of cells with agonists that trigger intracellular signalling cascades. Phosphorylation of IK1 channels or of associated proteins would then allow a fine tuning to the physiological need. Phosphorylation-dependent activation of IK1 channels occurs in native tissues and in heterologous expression systems. Several protein kinases have been shown to mediate Ca^{2+}-independent IK1-channel activation. Studies on cultured renal epithelial (MDCK) cells [27] suggested the involvement of protein kinase C (PKC). In fibroblasts, IK1 channels are regulated by mitogen-activated protein kinases [22]. The erythrocyte IK1 channel appears to be activated by PKC [16] and protein kinase A (PKA;

Table 1. Sequences of the primers used for mutagenizing putative PKC phosphorylation sites of cIK1

Mutant	Orientation	Primer sequence
T101	Sense	CGTGGCGCTGgCCGGGCGGCA
	Reverse	TGCCGCCCGGcCAGCGCCACG
S178	Sense	GCTCAACGCGgCCTACCGCAGC
	Reverse	GCTGCGGTAGGcCGCGTTGAGC
T329	Sense	CTACAAACACgCACGCAGGAAGG
	Reverse	CCTTCCTGCGTGcGTGTTTGTAG

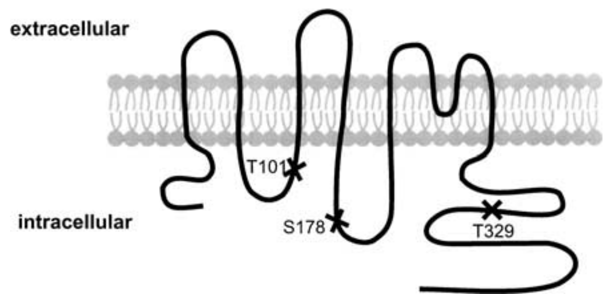

Fig. 1. This scheme indicates the positions of the mutated putative PKC phosphorylation sites within the cIK1 channel protein.

[20]). However, mutation of the single putative PKA phosphorylation site in the human isoform hIK1 does not alter its response to PKA agonists [7]. Further mutational analysis of hIK1 revealed a 14-amino-acid domain within its C-terminus that confers ATP- and phosphorylation-dependent regulation [8]. However, a definitive scheme of the molecular mechanism of this type of IK1-channel regulation has not yet been established. The involvement of yet unknown accessory channel subunits or other proteins interacting with IK1 channels appears likely.

Recently, we cloned the canine isoform of IK1 channels, cIK1, from migrating transformed MDCK-F cells (Schwab, submitted). In contrast to all other IK1 isoforms, cIK1 contains no putative PKA and only three PKC phosphorylation sites. Previously, we had shown that the native channel is regulated by PKC [27]. The present study was undertaken to shed light on the molecular mechanisms of PKC-dependent channel regulation.

Materials and Methods

Cell Culture

Experiments were carried out in CHO and HEK293 cells. Cells were kept at 37°C in humidified air containing 5% CO_2. CHO cells were grown in bicarbonate-buffered Minimal Essential Medium (MEM; pH 7.4) with Earle's salts (Biochrom, Berlin, Germany), and HEK293 cells in a 1:1 mixture of HamF12/DMEM (Dulbecco's Minimal essential medium) supplemented with 10% fetal calf serum. Culture medium of all stably transfected cells contained 0.6 g/l geneticin. Cells for patch-clamp experiments were always used 24–48 hr after they were plated at a density of 5×10^4 cells per 35-mm culture dish.

Site-Directed Mutagenesis

Site-directed mutagenesis of cIK1, which was subcloned into the EcoRV site of pcDNA3, was performed with PCR. First, two PCR products were generated that contained the desired mutation at their overlapping 3′ and 5′ ends, respectively. Table 1 summarizes the primers utilized. In a second step, these amplicons were fused by means of PCR. All mutations (cIK1-T101A, cIK1-S178A, cIK1-T329A) were verified by sequencing both cDNA strands. We generated three channel constructs in which only one putative PKC site was mutated at a time and one channel construct in which all three PKC sites were mutated simultaneously. Mutated cIK1 were only expressed in HEK293 cells. Fig. 1 indicates the positions of the mutated putative PKC phosphorylation sites within the channel protein.

Transfection of cIK1

Cells were transfected with Perfect Lipid (Pfx4, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Stably transfected CHO or HEK293 cells were selected by adding 0.6 g/l geneticin to the culture medium. Approximately 90% of these cells expressed wild-type or mutant cIK1 channels, respectively. Transiently transfected cells were cotransfected with pEGFP (Clontech, Palo Alto, CA) in order to visualize transfected cells by means of their green fluorescence. Cells were plated 24 hr after transient transfection at a density of 5×10^4 cells/ml onto 35-mm culture dishes.

Patch-Clamp Experiments

cIK1 and hIK1 currents were monitored with the patch-clamp technique in the whole-cell and inside-out patch configuration. Most experiments (unless stated differently) were performed in HEK293 cells. Patch pipettes (Hirschmann Laborgeräte, Eberstadt, Germany) had resistances between 2 and 4 MΩ when filled with the following solution (in mmol/l): 140 KC1, 0.77 $CaCl_2$, 1 $MgCl_2$, 1.3 EGTA, 10 HEPES, pH 7.4. Free intracellular Ca^{2+} varied between nominally Ca^{2+}-free and 10 μmol/l. Unless stated differently, the standard pipette solution contained a free Ca^{2+} concentration of 100 nmol/l. The bath solution contained (in mmol/l): 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4. Currents were recorded at room temperature with a List L/M-EPC7 patch-clamp amplifier (List, Medical Electronic, Darmstadt, Germany). Holding potential was usually −80 mV. The effect of drugs on cIK1 current was monitored by applying test pulses to +40 mV and steady-state mean current amplitudes were measured at this potential. Drugs were tested in paired experiments. Current amplitudes were determined when a new steady state was obtained under control conditions, after wash-in and wash-out of drugs, respectively. Data acquisition and current analysis were done with Pulse/Pulsefit software (HEKA Electronic, Lamprecht, Germany).

Statistics

Data are presented as mean ± SEM. We used Student's *t*-test or Mann-Whitney-U test for determining statistical significance ($p < 0.05$).

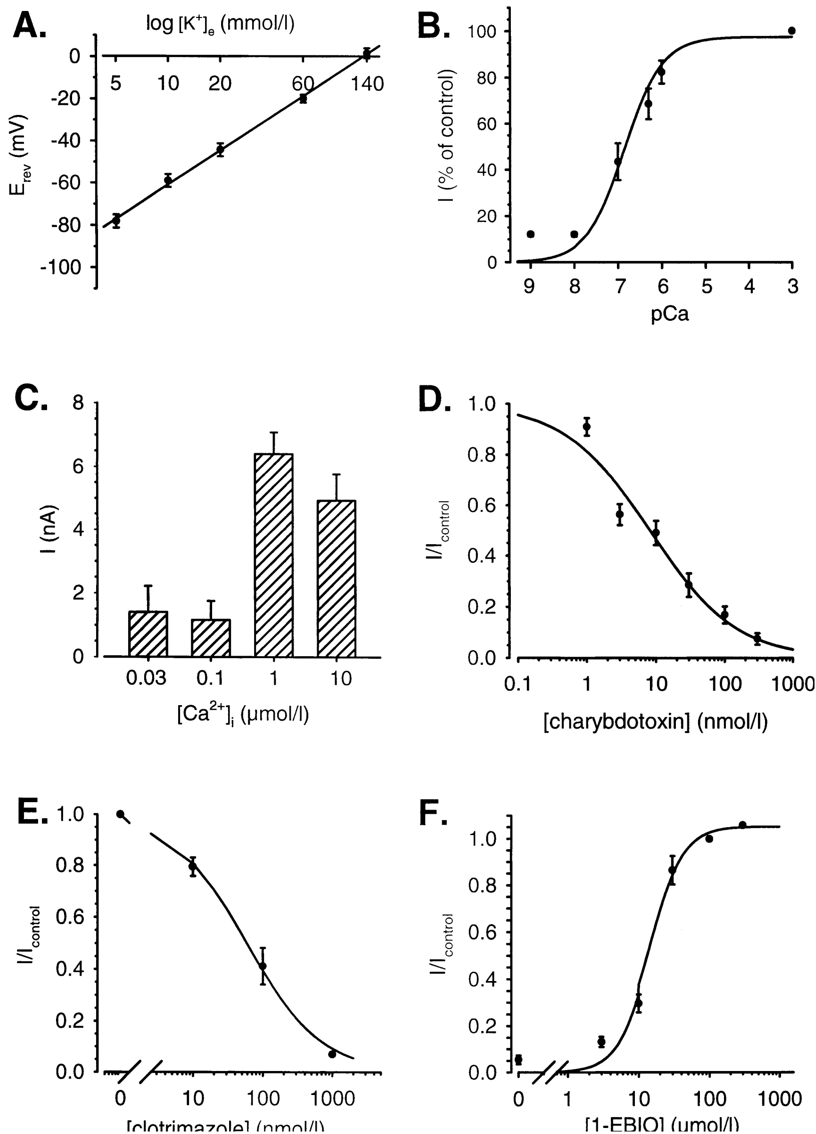

Fig. 2. Summary of important properties of cIK1 heterologously expressed in HEK293 cells. (*A*) cIK1 current is highly K^+ selective. A 10-fold change of the extracellular K^+ concentration shifts the reversal potential by 55 mV. cIK1 current is activated by Ca^{2+} in the inside-out patch configuration (*B*) and in the whole-cell configuration (*C*). In the presence of an intracellular Ca^{2+} concentration of 1 μmol/l, charybdotoxin (*D*) or clotrimazole (*E*) inhibit cIK1 current dose-dependently with an IC_{50} of 8.2 nmol/l and 59.5 nmol/l, respectively. (*F*) 1-EBIO activates cIK1 current half-maximally with a concentration of 14.9 μmol/l when the pipette solution contains 100 nmol/l Ca^{2+}.

Results

Properties of the Cloned cIK1 Channel

We ruled out that CHO or HEK293 cells express endogenous IK1 channels or channels that are sensitive to the drugs used in our study. The endogenous background current of single cells was usually less than 100 pA (at +40 mV). This is one to two orders of magnitude smaller than cIK1 or hIK1 current. Moreover, this current is not K^+-selective as judged from its reversal potential of approximately −10 mV in the presence of a physiological K^+ gradient. Whole-cell currents of cIK1-expressing CHO or HEK293 cells are not corrected for this endogenous background current.

cIK1 elicits a slightly inwardly rectifying current (Schwab, submitted) that is highly selective for K^+ over Na^+ (*see* Fig. 2*A*). Changing the extracellular K^+ concentration by a factor of 10 shifts the reversal potential of the whole-cell current by 54.9 mV. cIK1 is activated by an increase of the intracellular Ca^{2+} concentration. There is no cIK1 current with nominally Ca^{2+}-free solutions supplemented with 1.3 mmol/l EGTA. When studied in the inside-out patch configuration, cIK1 is activated by Ca^{2+} with a half-maximal concentration (EC_{50}) of 154 mmol/l (Fig. 2*B*). A similar result was obtained when cIK1 current was recorded in the whole-cell configuration with different intracellular Ca^{2+} concentrations (half-maximal inhibitory concentration IC_{50} = 102 nmol/l; Fig. 2*C*). cIK1 is inhibited by 1 mmol/l Ba^{2+} (*data not shown*), by charybdotoxin (IC_{50} = 8.2 nmol/l; Fig. 2*D*), and by clotrimazole (IC_{50} = 59.5 nmol/l; Fig. 2*E*), but it is not affected by 100 nmol/l iberiotoxin. Finally, cIK1 is activated by 1-ethyl-2-benzimidazolone (1-EBIO). When cIK1 is inactive before application of 1-EBIO (current amplitude smaller than 1 nA; *see* below), EC_{50} amounts to

14.9 µmol/l (*see* Fig. 2*F*). Overall, 1-EBIO served as a valuable tool in this study for assessing cIK1 expression in cells in which cIK1 was inactive. Taken together, these data indicate that cIK1 has similar properties as the human isoform hIK1 [9–11, 17].

Cloned cIK1 is Regulated by Protein Kinase C

There was no or only little cIK1 current (smaller than an arbitrarily chosen amplitude of 1 nA) in 8 out of 11 cIK1-expressing HEK293 cells when tested with nominally ATP-free pipette solution containing 100 nmol/l Ca^{2+}. However, the remaining cells (27%) had current amplitudes largely exceeding 1 nA. The higher current amplitude cannot be accounted for by a larger cell size. Mean membrane capacity, a measure of membrane surface, did not differ between these two cell populations. Average cIK1 current of all cells is 1.16 ± 0.6 nA (*see* Fig. 2*A*). A similar relation between "inactive" and "activated" cIK1 is found when cIK1 is expressed in CHO cells, and when the pipette solution contains only 30 nmol/l Ca^{2+} (3 out of 10 cells (30%) with cIK1 current equal or greater than 1 nA; mean current amplitude 1.4 ± 0.81 nA; *data not shown*).

When 1 mmol/l ATP was added to the pipette solution, the percentage of cells with activated cIK1 rose to 56% (mean current: 3.65 ± 2.1 nA) with 30 nmol/l Ca^{2+} and to 61% (mean current: 3.42 ± 0.7 nA) with 100 nmol/l Ca^{2+} in the pipette (*see* Fig. 3*A*). In order to test whether this activation depends on PKC we applied two structurally unrelated PKC inhibitors, bisindolylmaleimide (100 nmol/l; Calbiochem) and calphostin C (100 nmol/l; Calbiochem). Both drugs elicited a reversible and almost complete inhibition of cIK1 current (*see* Fig. 3*B*). Bisindolylmaleimide reduced cIK1 current to 4 ± 2% of control (n = 8) and calphostin, to 24 ± 4% (n = 6). These results point to a PKC-dependent regulation of heterologously expressed cIK1.

If phosphorylation is involved in modulating cIK1, this effect should be accentuated by adenosine-5′-[γ-thio]triphospate (ATP[γS]), an ATP analogue that mediates a phosphatase-resistant thio-phosphorylation. We therefore applied 2 mmol/l ATP[γS] to the pipette solution. All cells (6 out of 6) displayed current amplitudes exceeding 1 nA when the pipette contained 100 nmol/l Ca^{2+} (mean current 5.21 ± 0.25 nA; Fig. 3*A*), and 5 out of 6 did so with an intracellular Ca^{2+} concentration of 30 nmol/l (mean current 7.59 ± 1.97 nA). In the presence of ATP[γS], calphostin C lost its efficacy in inhibiting cIK1 (Fig. 3*C*). cIK1 current was reduced to only 70 ± 2% of control. This lends support to phosphorylation-dependent regulation of cIK1 and it is consistent with a de facto irreversible phosphorylation caused by

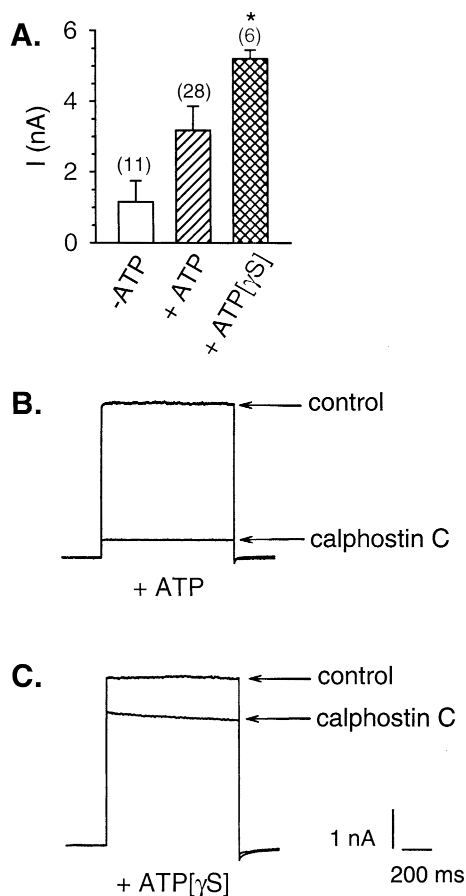

Fig. 3. (*A*) cIK1 current is increased when ATP or ATP[γS] is added to the pipette solution, which contains 100 nmol/l free Ca^{2+}. (*B*) ATP-induced cIK1 current is inhibited by the protein kinase C inhibitor calphostin C (100 nmol/l). (*C*) ATP[γS]-induced cIK1 current is only weakly inhibited by calphostin C, which is consistent with the practically irreversible phosphorylation mediated by ATP[γS]. *See* also Fig. 4.

ATP[γS]. Thus, the effect of PKC inhibitors on current amplitude is not due to a direct block of cIK1 channels. It probably reflects the action of phosphatases. However, the action of phosphatases is largely futile when cells are stimulated with ATP[γS].

If bisindolylmaleimide and calphostin C inhibit cIK1, stimulation of PKC should activate cIK1. We therefore applied phorbol 12-myristate 13-acetate (PMA, 100 nmol/l) to cells in which cIK1 was inactive (cIK1 current <1 nA). cIK1 current increased more than 11-fold. PMA stimulation of cIK1 current was reversed by BIM (Fig. 4). For comparison, we performed the same experiments with hIK1-expressing CHO cells. hIK1 was also activated by PMA, however, current amplitude rose only 2.3-fold. hIK1 stimulation was also reversed by bisindolylmaleimide (*data not shown*).

Collectively, these results indicate that heterologously expressed cIK1 is activated by PKC. Accordingly, downregulation of PKC by overnight

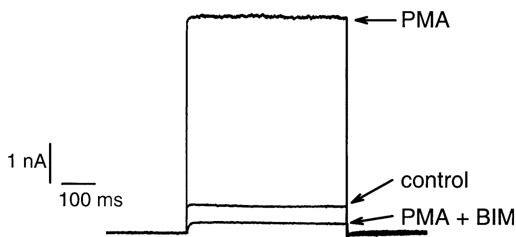

Fig. 4. The acute application of the phorbol ester phorbol 12-myristate 13-acetate (PMA, 100 nmol/l) elicits an 11-fold increase of cIK1 current. This effect is reversed by the protein kinase C inhibitor bisindolylmaleimide (BIM, 100 nmol/l).

incubation with 100 nmol/l PMA [15] abolished cIK1 activation by the addition of ATP to the pipette solution. cIK1 current exceeded 1 nA only slightly in one out of five cells (mean current of 0.53 ± 0.24 nA with 30 nmol/l Ca^{2+} and 0.83 ± 0.24 nA with 100 nmol/l Ca^{2+} in the pipette; *see* Fig. 6). The absence of cIK1 current is not due to retrieval of channel proteins from the plasma membrane. cIK1 current could still be elicited with 1-EBIO.

Finally, we also tested whether cIK1 (expressed in CHO cells) is modulated by protein kinase A. Therefore, we applied theophylline (1 mmol/l) and dibutyryl-cAMP (1 mmol/l) to the bath solution. This manouevre did not affect cIK1 current (0.23 ± 0.05 nA versus 0.32 ± 0.1 nA; *data not shown*).

Mutational Analysis of Putative PKC Phosphorylation Sites

We tested four cIK1 channel mutants in which either one putative PKC site at a time was mutated to alanine (T101A, S178A, T329A) or in which all three sites were mutated simultaneously (triple mutant). These mutants were tested in a similar way as described above for wild-type cIK1. All mutants formed functional channels. Their current-voltage curves were indistinguishable from wildtype cIK1 (*data not shown*). They also displayed the same Ca^{2+} sensitivity. In the absence of ATP, mutant cIK1 current saturated at 1 μmol/l Ca^{2+} (*data not shown*).

All four mutants exhibited a qualitatively similar response to the addition of ATP to the pipette solution. The percentage of cells with activated channels (current amplitude >1 nA) and/or the mean current amplitude rose. Current amplitudes of mutated cIK1's are not statistically different from that of wildtype cIK1 with ATP (cIK1-T101A: 5.96 ± 1.61 nA; cIK1-S178A: 3.41 ± 0.71 nA; cIK1-T329A: 4.13 ± 0.89 nA; cIK1 triple mutant: 4.35 ± 1.08 nA). Fig. 5 shows that current of all cIK1 mutants was inhibited by bisindolylmaleimide or calphostin C to the same extent in the presence of ATP as was that of wildtype cIK1 (cIK1-T101A: 22 ± 16%; cIK1-S178A: 19 ± 6%; cIK1-T329A: 23 ± 5%; cIK1 triple mutant: 21 ± 2%). The sensitivity to PKC inihbitors was reduced like that of wildtype cIK1 when cIK1 mutants were activated by ATP[γS]. Current was reduced to only approximately 80% of control (cIK1-T101A: 90 ± 9%; cIK1-S178A: 77 ± 5%; cIK1-T329A: 81 ± 7%; triple mutant: 88 ± 3%). The only difference is that the current amplitudes of cIK1-T329A and of the triple mutant in the presence of ATP[γS] were larger than those of wildtype cIK1 and of the other two cIK1 mutants (approximately 8 nA versus 5 nA). Finally, we also tested the effect of the acute PKC stimulation with 100 nmol/l; PMA. All mutants could be activated by PMA. cIK1 current was potentiated at least 6-fold (*data not shown*). Collectively, these data indicate that the three mutated putative PKC phosphorylation sites (T101, S178, T329) are not involved in the acute activation of cIK1 by protein kinase C.

This is in contrast to the effect of overnight incubation with PMA (Fig. 6). When the putative PKC site T329 is mutated to alanine the cIK1 channel behaves differently. The mutants cIK1-T101A and cIK1-S178A responded with inactivation of channel activity after long-term treatment with PMA. Mean current amplitudes were: cIK1-T101A: 1.73 ± 1.41 nA; cIK1-S178A: 0.82 ± 0.36 nA. This is analogous to wildtype cIK1. cIK1 mutant T329A, however, was highly activated (mean current amplitude of 11.01 ± 1.01 nA). There was no other maneuver that produced such consistently high current amplitudes. When all three putative PKC sites were mutated, long-term PMA treatment also led to cIK1 activation (mean current amplitude of 6.9 ± 1.46 nA). Both, cIK1-T329A and the triple mutant, lost their sensitivity to PKC inhibition after overnight treatment with PMA. Bisindolylmaleimide reduced current amplitude only by 7% (cIK1-T329A) or 11% (cIK1 triple mutant). This indirectly confirms that PKC activity is virtually absent after long-term treatment with PMA and that activation of cIK1-T329A is a consequence of PKC down modulation [15]. These data are consistent with a second inhibitory mechanism by which the PKC site at T329 exerts a direct effect on cIK1. When the PKC site is mutated, this inhibitory effect is abolished and cIK1 current amplitude is elevated.

Discussion

Our study provides evidence that protein kinase C plays an important role in the regulation of heterologously expressed cIK1 channels. Both, PKC inhibitors and activators, elicit inhibitory and stimulatory effects on cIK1 channels, respectively. Thus, PKC and an increase of the intracellular Ca^{2+} concentration act synergistically on cIK1 activity. This is in good agreement with experiments performed with

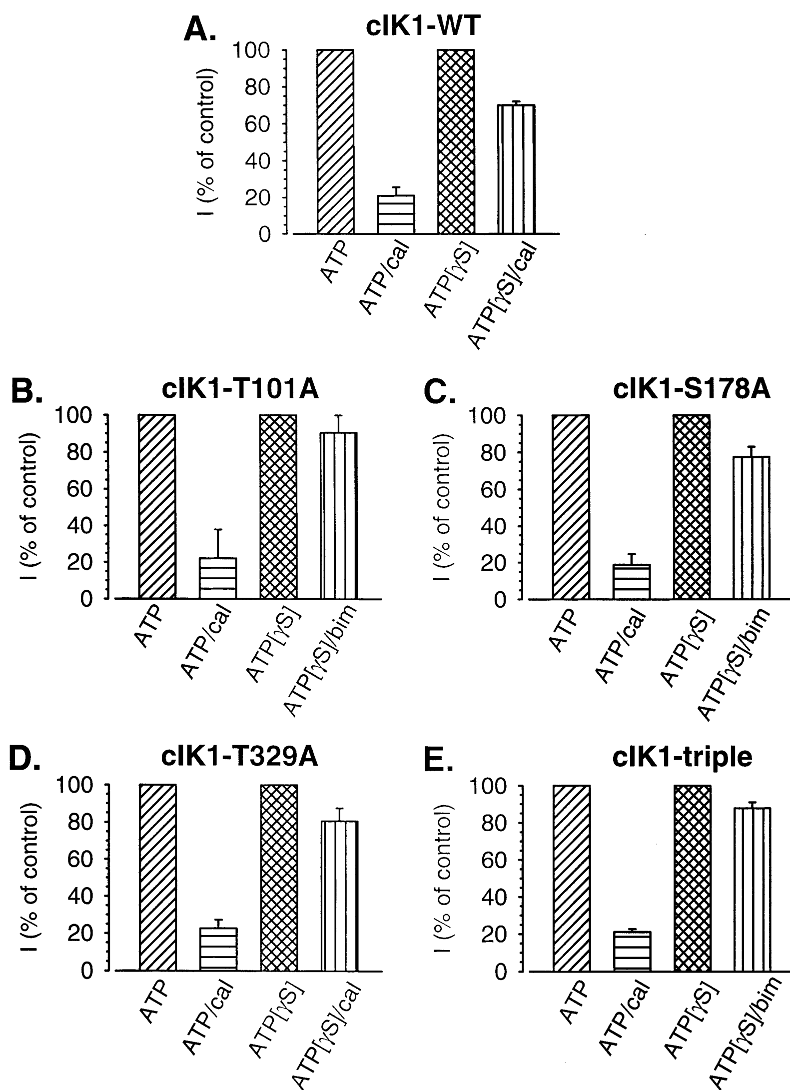

Fig. 5. Effect of protein kinase C inhibitors on ATP- or ATP[γS]-induced cIK1 current. (*A*) wild-type cIK1. (*B–E*) mutated cIK1 channels. All mutated cIKl channels react in the same way to PKC inhibitors as does wild-type cIK1. ATP-induced currents are inhibited almost completely, whereas ATP[γS]-induced currents are largely resistant to PKC inhibition. In all cases, the concentration of calphostin and BIM was 100 nmol/l, respectively.

native cIK1 in MDCK cells [27]. PKC-mediated activation renders cIK1 practically insensitive to the intracellular Ca^{2+} concentration in the physiological range. We measured the same cIK1 current amplitude when the pipette solution contained 1 μmol/l Ca^{2+} or 30 nmol/l Ca^{2+} combined with ATP[γS], Like native cIK1, the cloned channel is not regulated by protein kinase A. We failed to activate cIK1 expressed in CHO cells with a combination of 8-bromo-cAMP and theophylline. This behavior is in contrast to the human isoform hIK1, which was shown to be responsive to PKA and/or xanthin derivatives [7, 25; unpublished obervations from our laboratory]. Interestingly, the lack of PKA sensitivity of cIK1 is paralleled by the absence of a putative PKA consensus phosphorylation site in the cIK1 channel protein. However, it has been shown that this consensus site is not the target of PKA-dependent regulation of hIK1 [7, 25].

It is conspicuous that ATP does not produce a uniform response with respect to cIK1 channel activation. ATP elicits cIK1 current exceeding 1 nA in only approximately two out of three cells. Several reasons may account for this behavior. Our cells were not clonal since they were not isolated by single-cell dilution after transfection with wild-type or mutant cIK1. Thus, the heterogeneous response to ATP might be related to differences between individual cells. However, this seems unlikely in our view since the ratio of cells with active and inactive cIK1 channels does not vary between different cIK1-transfected stable cell lines. The heterogeneous response to ATP may also be a technical artifact. All experiments were performed in the whole-cell configuration of the patch-clamp technique, which involves the dialysis of the cytoplasm. Therefore, we cannot exclude that essential components of the PKC signalling cascade were lost in some cells. Finally, the variable response to ATP can reflect the fact that IK1 channels are regulated by a number of interrelated intracellular signalling pathways. IK1 channels in fibroblasts, for example, are upregulated by basic fibroblast growth

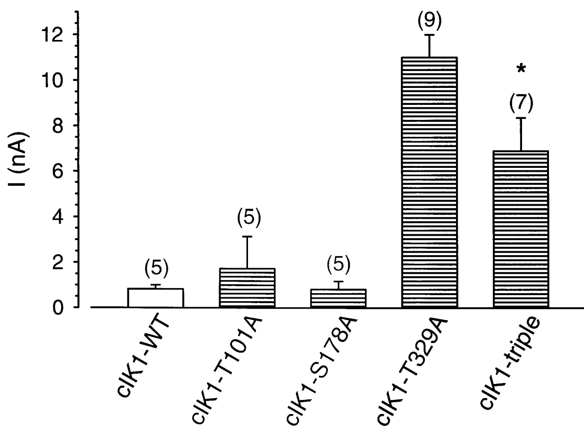

Fig. 6. Effect of PKC depletion by longterm incubation with the phorbol ester PMA on current of wild-type and mutated cIK1 channels. The pipette solution contained 100 nmol/l Ca^{2+} and 1 mmol/l ATP. cIK1-T329A and the cIK1 triple mutant respond with a massive increase of current amplitude to this treatment.

factor (bFGF). bFGF action requires the Ras/mitogen-activated protein (MAP) kinase/MAP kinase ERK-kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway [22]. In this study, IK1 current was only determined after prolonged exposure to bFGF, and the acute effect of bFGF was not mentioned. The MAPK/ERK pathway can be activated by protein kinase C [24]. Due to this crosstalk PKC-dependent cIK1 channel activation could also involve a more rapid action of the MAP kinase pathway. This notion is supported by our finding that an inhibitor of ERK phosphorylation (PD98059) acutely blocks ATP-dependent cIK1 current in HEK293 cells (unpublished observations from our laboratory). Presently, we do not know whether and, if so, how PKC and MAPK/ERK pathways are to be coordinated in order to allow maximal cIK1 channel activation. However, it is conceivable that cell cycle-dependent variations of their activation can also contribute to the heterogeneous ATP response.

The mutational analysis of three conserved putative PKC phosphorylation sites in the cIK1 channel protein reveals that neither of them alone nor the combination of all three appear to be involved in the acute activation of cIK1 by PKC. None of the mutants displays a qualitatively altered response to ATP[γS], PKC inhibitors or acute stimulation with PMA. These results provide evidence that cIK1 channels are probably not the direct target of the acute effects of PKC. PKC rather activates cIK1 channels in an indirect way. The proteins that eventually transmit the stimulatory effect of PKC onto the channel protein are not yet known. A similar conclusion was reached for the human isoform hIK1, since mutation of putative phosphorylation sites does not modify ATP-dependent channel gating [8].

However, our study provides evidence for a second way by which cIK1 channels can be regulated, and this effect seems to depend on a direct interaction with the channel protein. Mutation of the putative PKC site T329 unmasks an apparently paradox stimulatory effect (or disinhibition) of PKC depletion on cIK1 current. No other maneuver elicited with such a regularity currents of this amplitude. This is not due to a different basal cIK1 expression level. First, basal cIK1 current of the T329A mutant is only slightly higher than that of wild-type cIK1 or the cIK1-T101A and cIK1-S178A mutants. Second, the triple mutant reproduces the behavior of the cIK1-T329A mutant. We also do not believe that increased current amplitude of the cIK1-T329A or the triple mutant are caused exclusively by a modification of channel gating. Ca^{2+} sensitivity and the effects of acute PKC inhibition or stimulation are indistinguishable between wild-type cIK1 and the cIK1-T329A or cIK1 triple mutants. Finally, it is unlikely that PMA treatment selectively upregulates transcription of the heterologously expressed cIK1-T329A or cIK1 triple mutants while leaving wild-type cIK1 and the other two cIK1 mutants unaffected, which are also heterologously expressed. Such transcriptional upregulation was shown for native hIK1 channels during T lymphocyte activation [6].

We envisage another explanation, although we are aware that it is rather speculative at this moment and requires further experimental verification. The PKC site T329 is in close vicinity of a conserved dileucine motiv. Dileucine motifs are involved in targeting membrane proteins to the basolateral membrane of polarized epithelia [12]. This also applies for hIK1 channels (Schwab, submitted). Moreover, dileucine motifs play an important role in the endocytotic internalization of membrane proteins such as the CD3γ subunit of the T cell receptor or the acetylcholine transporter. Internalization of these membrane proteins requires PKC-dependent phosphorylation in the close vicinity of the dileucine motif. Mutating the respective PKC phosphorylation sites impedes their endocytic internalization [4, 14]. Along these lines, the increased current amplitude of the cIK1-T329A and the cIK1 triple mutant could be a consequence of disturbed membrane trafficking. Mutated cIK1 channels are internalized at a decreased rate and remain in the plasma membrane, thereby increasing the current amplitude. This could account for the increased current amplitude of cIK1-T329A and the triple mutant in response to ATP[γS]. This effect is further accentuated when PKC-dependent protein phosphorylation, which is required for endocytosis [3], is impaired after PKC depletion with phorbol esters. However, we do not dismiss the possibility that the relatively unspecific maneuver of PKC depletion can induce activation of the cIK1-T329A or cIK1 triple mutants by other, as of yet

unrecognized, ways. For example, the activation of cIK1-T329A could also be explained by an increased ATP sensitivity leading to altered channel gating.

The concept of PKC-modulated cIK1 internalization could account for the observed increase in current amplitude of the cIK1-T329A mutant. However, we must emphasize that it only provides the framework for the experimental verification (by means of biochemical and/or fluorescence microscopy techniques) of this type of membrane trafficking of the cIK1 channel protein and that several open questions remain. Amongst others, the time course of PKC-modulated channel internalization needs to be determined in more detail. Is it as fast as endocytic retrieval at the synaptic membrane [19], or does it follow a slower time course? Our results point towards a kinetics that is usually slower than that of the acute effect of PKC on channel activity. Otherwise, PKC-dependent stimulation would have been greater in the cIK1-T329A mutant relative to cIK1 wildtype. We observed a biphasic response to PMA in one cell when we monitored cIK1 wildtype current for an extended period of time. cIK1 current rose to a maximum within 3 min (23-fold increase) and then gradually declined within the next 6 min by 28% (to the 17-fold amplitude of control current). PKC-modulated endocytic internalization and recycling of IK1 channels can be physiologically meaningful in conditions of high membrane turnover, which commonly occur in migrating cells [1]. Such a mechanism would ensure that IK1 channels are kept at their "place of work", i.e., in the plasma membrane, where they exert their physiological functions.

We thank Barbara Schuricht and Birgit Gassner for expert technical assistance. This work was supported by Deutsche Forschungsgemeinschaft Schw 407/7-1.

References

1. Aguado-Velasco, C., Bretscher, M.S. 1999. Circulation of the plasma membrane in *Dictyostelium*. *Mol. Biol. Cell* **10:**4419–4427
2. Bleich, M., Riedemann, N., Warth, R., Kerstan, D., Leipziger, J., Hör, M., Van Driesche, W., Greger, R. 1996. Ca^{2+}-regulated K^{+} and non-selective cation channels in the basolateral membrane of rat colonic crypt base cells. *Pflügers Arch.* **432:**1011–1022
3. Cousin, M.A., Tan, T.C., Robinson, P.J. 2001. Protein phosphorylation is required for endocytosis in nerve terminals: potential role for the dephosphins dynamin I and synaptojanin, but not AP180 or amphiphysin. *J. Neurochem.* **76:**105–116
4. Dietrich, J., Hou, X., Wegener, A.-M.K., Geisler, C. 1994. CD3γ contains a phosphoserine-dependent di-leucine motif involved in down-regulation of the T cell receptor. *EMBO J.* **13:**2156–2166
5. Fanger, C.M., Ghanshani, S., Logsdon, N., Rauer, H., Kalman, K., Zhou, J., Beckingham, K., Chandy, K.G., Cahalan, M.D., Aiyar, J. 1999. Calmodulin mediates the calcium-dependent activation of the intermediate conductance K_{ca} channel, IKCa1. *J. Biol. Chem.* **274:**5746–5754
6. Ganshani, S., Wulff, H., Miller, M.J., Rohm, H., Neben, A., Gutman, G.A., Cahalan, M.D., Chandy, K.G. 2000. Up-regulation of the IKCa1 potassium channel during T-cell activation. *J. Biol. Chem.* **275:**37137–37149
7. Gerlach, A.C, Gangopadhyay, N.N., Devor, D.C. 2000. Kinase-dependent regulation of the intermediate conductance calcium-dependent potassium channel, hIK1. *J. Biol. Chem.* **275:**585–598
8. Gerlach, A.C., Syme, C.A., Giltinan, L., Adelman, J.P., Devor, D.C. 2001. ATP-dependent activation of the intermediate conductance, Ca^{2+}-activated hIK1, is conferred by a C-terminal domain. *J. Biol. Chem.* **276:**10963–10970
9. Ishii, T.M., Silvia, C., Hirschberg, B., Bond, C.T., Adelman, J.P. 1997. A human intermediate conductance calcium-activated potassium channel. *Proc. Natl. Acad. Sci.* **94:**11651–11656
10. Jensen, B.S., Strøbaeck, D., Christophersen, P., Jørgensen, T.D., Hansen, C., Silahtaroglou, A., Olesen, S.-P., Ahring, P.K. 1998. Characterization of the cloned human intermediate-conductance Ca^{2+}-activated K^{+} channel. *Am. J. Physiol.* **275:**C848–C856
11. Joiner, W.J., Wang, L.-Y., Tang, M.D., Kaczmarek, L.K. 1997. hSK4, a member of a novel subfamily of calcium-activated potassium channels. *Proc. Natl. Acad. Sci.* **94:**11013–11018
12. Keller, P., Simons, K. 1997. Post-Golgi biosynthetic trafficking. *J. Cell. Sci.* **110:**3001–3009
13. Köhler, R., Degenhardt, C., Kühn, M., Runkel, N., Paul, M., Hoyer, J. 2000. Expression and function of endothelial Ca^{2+}-activated K^{+} channels in human mesenteric arteries. *Circ. Res.* **87:**496–503
14. Krantz, D.E., Waites, C., Oorschot, V., Liu, Y., Wilson, R.L, Tan, P.K., Klumperman, J., Edwards, R.H. 2000. A phosphorylation site regulates sorting of the vesicular acetylcholine transporter to dense core vesicles. *J. Cell. Biol.* **149:**379–395
15. Krug, E., Bieman, H.-P., Tashjian, A.H. 1987. Evidence for increased synthesis as well as increased degradation of protein kinase C after treatment of human osteosarcoma cells with phorbol esters. *J. Biol. Chem.* **262:**11852–11856
16. Lew, V.L., Ferreira, H.G. 1976. Variable Ca sensitivity of a K selective channel in intact red-cell membranes. *Nature* **263:**336–338
17. Logsdon, N.J., Kang, J., Togo, J.A., Christian, E.P., Aiyar, J. 1997. A novel gene, hKCA4, encodes the calcium-activated potassium channel in human T lymphocytes. *J. Biol. Chem.* **272:**32723–32726
18. Meyer, R., Schönherr, R., Gavrilova-Ruch, O., Wohlrab, W., Heinemann, S. 1999. Indentification of *ether à go-go* and calcium-activated potassium channels in human melanoma cells. *J. Membrane Biol.* **171:**107–115
19. Moser, T., Beutner, D. 2000. Kinetics of exocytosis and endocytosis at the cochlear inner hair cell afferent synapse of the mouse. *Proc. Natl. Acad. Sci.* **97:**883–888
20. Pellegrino, M., Pellegrini, M. 1998. Modulation of Ca^{2+}-activated K^{+} channels of human erythrocytes by endogenous cAMP-dependent protein kinase. *Pflügers Archiv Eur. J. Physiol.* **436:**749–756
21. Peña, T.L, Rane, S.G. 1999. The fibroblast intermediate conductance K_{Ca} channel, FIK, as a prototype for the cell growth regulatory function of the IK channel family. *J. Membrane Biol.* **172:**249–257
22. Peña, T.L, Chen, S.H., Konieczny, S.F., Rane, S.G. 2000. Ras / MEK / ERK up regulation of the fibroblast K_{Ca} channel FIK is a common mechanism for basic fibroblast growth factor and transforming growth factor-ß suppression of myogenesis. *J. Biol. Chem.* **275:**13677–13682

23. Schmid-Antomarchi, H., Schmid-Alliana, A,. Romey, G., Ventura, M.-A., Breittmayer, V., Millet, M.-A., Husson, H., Moghrabi, B., Lazdunki, M., Rossi, B. 1997. Extracellular ATP and UTP control the generation of reactive oxygen intermediates in human macrophages through the opening of a charybdotoxin-sensitive Ca^{2+}-dependent K^{+} channel. *J. Immunol.* **159:**6209–6215
24. Schönwasser, D.C, Marais, R.M, Marshall, C.J., Parker, P.J. 1998. Activation of mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. *Mol. Cell. Biol.* **18:**790–798
25. Schrøder, L., Jensen, B.S., Strøbseck, D., Oleson, S.-P., Christophersen, P. 2000. Activation of the human, intermediate-conductance, Ca^{2+}-activated K^{+} channel by methylxanthines. *Pflügers Arch. Eur. J. Physiol.* **440:**809–818
26. Schwab, A. 2001. Function and spatial distribution of ion channels and transporters in cell migration. *Am. J. Physiol.* **280:**F739–F747
27. Schwab, A., Geibel, J., Wang, W., Oberleithner, H., Giebisch, G. 1993. Mechanism of activation of K^{+} channels by Minoxidil-sulfate in Madin-Darby canine kidney cells. *J. Membrane Biol.* **132:**125–36
28. Schwab, A., Reinhardt, J., Schneider, S.W., Gaßner, B., Schuricht, B. 1999. K^{+} channel dependent migration of fibroblasts and human melanoma cells. *Cell. Physiol. Biochem.* **9:**126–132
29. Schwab, A., Reinhardt, J., Seeger, P., Schuricht, B., Dartsch, P.C. 1999. Migration of transformed renal epithelial cells is regulated by K^{+} channel modulation of actin cytoskeleton and cell volume. *Pflügers Arch. Eur. J. Physiol.* **438:**330–337
30. Schwab, A., Wojnowski, L., Gabriel, K., Oberleithner, H. 1994. Oscillating activity of a Ca^{2+}-sensitive K^{+} channel—a prerequisite for migration of alkali-transformed Madin-Darby canine kidney (MDCK-F) cells. *J. Clin. Invest.* **93:**1631–1636
31. Warth, R., Hamm, K., Bleich, M., Kunzelmann, K., von Hahn, T., Schreiber, R., Ullrich, E., Mengel, M., Trautmann, N., Kindle, P., Schwab, A., Greger, R. 1999. Molecular and functional characterization of the small Ca^{2+} activated K^{+} channel (rSK4) of colonic crypts. *Pflügers Arch. Eur. J. Physiol.* **438:**437–444
32. Varnai, P., Demaurex, N., Jaconi, M., Schlegel, W., Lew, D.P., Krause, K.H. 1993. Highly cooperative activation of intermediate-conductance K^{+} channels in granulocytes from a human cell line. *J. Physiol.* **472:**373–390