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KCl Cotransport Regulation and Protein Kinase G in Cultured Vascular Smooth Muscle Cells

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Abstract. K-Cl cotransport is activated by vasodilators in erythrocytes and vascular smooth muscle cells and its regulation involves putative kinase/phosphatase cascades. N-ethylmaleimide (NEM) activates the system presumably by inhibiting a protein kinase. Nitrovasodilators relax smooth muscle via cGMP-dependent activation of protein kinase G (PKG), a regulator of membrane channels and transporters. We investigated whether PKG regulates K-Cl cotransport activity or mRNA expression in normal, PKG-deficient-vector-only-transfected (PKG−) and PKG-catalytic-domain-transfected (PKG+) rat aortic smooth muscle cells. K-Cl cotransport was calculated as the Cl-dependent Rb influx, and mRNA was determined by semiquantitative RT-PCR. Baseline K-Cl cotransport was higher in PKG+ than in PKG− cells ($p < 0.01$). At 0.5 mM, NEM stimulated K-Cl cotransport by 5-fold in PKG− but not in PKG+ cells. However, NEM was more potent although less effective to activate K-Cl cotransport in normal (passage 1–3) and PKG+ than in PKG− cells. In PKG− cells, [(dihydroindenyl) oxy] alkanoic acid (300 μM) but not furosemide (1 mM) inhibited K-Cl cotransport. Furthermore, no difference in K-Cl cotransport mRNA expression was observed between these cells. In conclusion, this study shows that manipulation of PKG expression in vascular smooth muscle cells affects K-Cl cotransport activity and its activation by NEM.

Key words: K-Cl cotransport — Protein kinase G — Vascular smooth muscle cells — N-ethylmaleimide — Furosemide — [(dihydroindenyl) oxy] alkanoic acid — mRNA expression

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Introduction

Abnormalities in monovalent ion transport homeostasis have been observed in various tissues and are associated with cardiovascular disease (Orlov et al., 1999). K-Cl cotransport (COT), the electroneutral coupled movement of K and Cl, plays an important role in cellular Cl and K homeostasis (Brugnara, Van Ha & Tosteson, 1989; Lauf et al., 1992; Kaji, 1993; Lauf & Adragna, 1996; Lauf & Adragna, 2000). N-ethyl maleimide (NEM), a thiol reagent, is one of the most commonly used activators of K-Cl COT (Lauf & Theg, 1980), although its mechanism of action has not been identified. Likewise, the regulation of the system is largely undefined. For example, stimulation of K-Cl COT by SH-reagents such as NEM and diamide (Lauf et al., 1992; Lauf & Adragna, 2000), and by nitrite (Adragna & Lauf, 1998) and several other vasodilators (Lauf et al., 1992; Adragna et al., 1999, 2000; Lauf & Adragna, 2000) present a characteristic lag phase that has been linked to regulation by a kinase/phosphatase system (Jennings & al-Rohil, 1990; Starke & Jennings, 1993; Jennings, 1999). Okadaic acid (Orringer et al., 1991) and calyculin A, are inhibitors of serine-threonine protein phosphatases, and genistein is a tyrosine kinase inhibitor (Flatman, Adragna & Lauf, 1996). These drugs inhibit K-Cl COT activation by NEM, suggesting that a regulatory cascade of kinases and phosphatases might be involved in K-Cl COT regulation (Jennings & al-Rohil, 1990; Starke & Jennings, 1993; Bize & Dunham, 1994; Flatman et al., 1996; Jennings, 1999). Indeed, a correlation between serine/threonine protein phosphatases and K-Cl COT activities has been recently reported for human RBCs (Bize et al., 1999).

So far, four genes (KCC1-KCC4) and two splice variants, KCC3a and b, of the transporter have been

described (*see* references in Lauf & Adragna, 2000), all members of the superfamily of cation-chloride cotransporters (Shmukler, Brugnara & Alper, 2000). The five KCC isoforms have been described and characterized in several tissues (Gillen et al., 1996; Hiki et al., 1999; Su et al., 1999), including vascular smooth muscle cells (VSMCs) (Di Fulvio et al., 2001).

We recently reported that K-Cl COT is present in VSMCs, that it is activated by NO donors and that this effect is prevented by compounds interfering with the cGMP pathway (Adragna & Lauf, 1998; Adragna, Ahmed & Lauf, 1998; Adragna et al., 1999, 2000). This pathway involves the serine/threonine protein kinase G, PKG. Thus, we hypothesized that NO regulates vascular smooth muscle K-Cl COT by activating PKG and modulating either the mRNA expression, the activity of the transporter or both. However, cGMP can regulate cellular function by activation of PKG or by acting directly on nonselective ion channels, such as in the vertebrate rod photoreceptor cell as well as renal and olfactory epithelial channels (Bolotina et al., 1994) and the Ca-dependent K channels in VSMCs (Lincoln, 1994).

In VSM, PKG is involved in several mechanisms that are crucial for tissue and body functions (Lincoln, 1994). Two types I PKG isoforms, Iα and Iβ, are the result of the alternative splicing of a unique gene (Wernaet, Flockerzi & Hofmann, 1989). These 75 kDa forms differ only at the amino terminus, but share the same carboxy terminus, wherein the cGMP-binding sites and the catalytic domain reside (Gudi et al., 1996; Boerth et al., 1997; Surks et al., 1999). Regulation of PKG expression in VSMCs is largely undefined (Lincoln et al., 1998). However, both, repetitive passage of cultured rat aortic SMCs and continuous exposure of cultured bovine aortic SMCs to NO donors, are associated with reduced to nearly undetectable expression levels of PKG (Boerth et al., 1997; Soff et al., 1997). Expression of PKG is also dependent on VSMC density (Cornwell et al., 1994). The PKG content of VSMCs determines their phenotype. Thus, PKG-containing cells possess a contractile-like morphology, whereas PKG-deficient cells become dedifferentiated and "synthetic" (Boerth et al., 1997). Transfection of PKG-deficient VSMCs with cDNAs encoding the full-length PKG I or its carboxy-terminal catalytic domain reverts the transfected cells to their original contractile morphology (Boerth & Lincoln, 1994). On the other hand, in PKG I-deficient mice, the loss of PKG I abolishes NO/cGMP-dependent relaxation of VSM, resulting in severe vascular dysfunction. However, PKG I-deficient VSM responds normally to cAMP, indicating that cAMP and cGMP signal via independent pathways, with PKG I being the specific mediator in NO/cGMP effects in VSM (Pfeifer et al., 1998).

Presently, our laboratory is focusing on the study of the role of NO, cGMP and PKG on K-Cl COT regulation (Adragna et al., 1998, 1999; Adragna & Lauf, 1998**;** Adragna et al., 2000, 2001; Di Fulvio et al., 2001). Because cGMP may activate PKG-independent processes (*see* above), we used primary cultures of normal rat aortic SMCs (RASMCs) and tested a cellular model of RASMCs deficient in endogenous PKG and transfected with either a vector alone (PKG−) or with a vector containing the catalytic domain of PKG (PKG+) (Boerth & Lincoln, 1994). To further understand the role of PKG on K-Cl COT regulation, we investigated the effect of the known activator NEM, and also the known inhibitors furosemide and [(dihydroindenyl)oxy]alkanoic acid (DIOA) on K-Cl COT activity and expression (Lauf et al., 1992; Orlov et al., 1992; Adragna et al., 2000; Lauf & Adragna, 2000; Di Fulvio et al., 2001). In the present model, differences in the behavior of PKG− and PKG+ cells with respect to K-Cl cotransport modulation could be more clearly attributed to a NO/cGMP/PKG mediated process.

Part of this work has been presented elsewhere in abstract form (Adragna et al., 2001).

Materials and Methods

Materials

DMEM medium, tissue culture grade and molecular biology reagents were purchased from Gibco BRL (Gaithersburg, MD). Total RNA extractions, and PCR fragments purification kits, were from Promega Corp.

Primary Culture of Rat VSMCs

Primary cultures of rat VSMCs were done according to the protocols described previously (Boerth et al., 1997; Soff et al., 1997) with modifications. Briefly, aortas from Sprague Dawley rats (150–200g), sacrificed by CO_2 inhalation were provided by the Wright State University Animal Facilities. Aortas were excised and washed in DMEM with 20 mM HEPES, 1 mg/ml bovine serum albumin, 5 amphotericin B, and 50 μg/ml gentamicin. The aortas were rinsed and placed in digestion medium (130 U/ml collagenase type IV, 5 μg/ml DNAase I), 10 min at 37°C. The tunica adventitia was removed, and the medial layers were minced and further digested for 1–2 hr in digestion medium containing 200 U/ml collagenase until a single-cell suspension was obtained. Cells were washed twice in DMEM containing 10% fetal bovine serum and antibiotics [streptomycin (100 μg/ml), penicillin (100 U/ml), gentamicin (50 μg/ml) and amphotericin B (2.5 μg/ml)], and plated in 12-well plates. Cells were maintained in DMEM plus 10% fetal bovine serum in a controlled atmosphere of air-CO_2 (5%) at 37°C until confluence (6–7 days).

Culture of PKG− and PKG+ RASMCs

RASMCs, transfected with either vector alone or the active-catalytic domain of PKG I, which lacks the cGMP-binding site (Boerth & Lincoln, 1994), were cultured in the same culture medium and conditions as normal RASMCs, but in the presence of 10% CO_2 and 500 μg/ml geneticin (G418) (Boerth & Lincoln, 1994). Cells were allowed to reach about 85% confluence. The culture medium

was changed every 48–72 hr during 5–6 days, at which time the desired level of confluence was normally reached. At least three independent clones of normal, PKG− and PKG+ RASMCs were routinely examined.

Cell Transfection

The catalytic domain of bovine PKG−Iα cDNAs was constructed as indicated elsewhere (Boerth & Lincoln, 1994; Boerth et al., 1997). RASMCs at passage 4, which are deficient in endogenous PKG I, were exposed to 5 μg of recombinant or control plasmid DNA using Transfectam reagent with precipitation of the DNA-liposome complex for 15 min at room temperature. The precipitate was added for 6 hr to the cell monolayer growing at 37°C and 10% CO_2. The transfection process was finished by adding fresh culture medium supplemented with 20% fetal bovine serum. Stably transfected cells were selected using 500 μg/ml G418 antibiotic and isolated from 96-well plates. The transfected VSMCs were maintained in 250 μg/ml G418 medium.

Total RNA Extraction, RT-PCR and KCC Expression in VSMCs

Total RNA from VSMCs was obtained according to Chomczynski and Sacchi (1987). Specific sets of primers for all KCC mRNA isoforms were synthesized according to the sequences previously published by others (Gillen et al., 1996; Hiki et al., 1999; Mount et al., 1999). These primers were used to obtain the first cDNA strand by RT, and the subsequent amplification of each KCC isoform present in VSMC was done by PCR as previously described (Di Fulvio et al., 2001). The semiquantitative RT-PCR conditions were established in our laboratory in order to allow comparisons between the expression of the KCC1 and KCC3 transcripts. Under these conditions, the efficiency of the RT-PCR reaction for each gene did not plateau and the numbers of cycles used in these experiments were kept to a minimum (Di Fulvio et al., 2001). The relative expression levels of KCC1 and KCC3 isoforms was determined by using 1.0 μg total RNA as template and 30 cycles of PCR with the same thermal conditions as before. As control, we analyzed the expression of actin mRNA using specific rat primers (Promega), the same amount of total RNA as before, and 25 PCR cycles. These were optimal conditions for the semiquantitative analysis of VSMC KCC mRNAs (Di Fulvio et al., 2001). The general semiquantitative RT-PCR protocol was as follows: RT-PCR reactions were performed preparing a master RT-PCR mix containing 3.0 μg of total RNA from VSMCs (normal, PKG− and PKG+, treated/untreated with agents) and divided in 3 separate tubes containing the specific set of primers for KCC1, KCC3, PKG− and actin, respectively. As a negative control for each set of primers, RT-PCR reactions were performed in the absence of reverse transcriptase. After RT-PCR, the content of each independent reaction tube was analyzed by 2% agarose gel electrophoresis. The bands (KCC1, 233 bp; KCC3, 663 bp; and actin, 285 bp) were visualized with ethidium bromide. All the ethidium bromide-stained gels were depicted as an inverse image for clear results. The identity of KCC1 and KCC3 was confirmed by restriction enzyme digestion according to the sequences published and the expected DNA fragments obtained.

K-Cl COT Measurement

Cells were grown for 3–7 days (80–100% confluence) on 12-well plates in DMEM growth medium and assayed for Rb influx in balanced salt solution (BSS) containing (mM): 130 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 20 HEPES/Tris buffered to pH 7.4 at 37°C. Cl-free solutions contained Na and K sulfamate and Ca and Mg gluconate salts. Due to the lack of a specific inhibitor of K-Cl COT, inhibitors of other pathways for K/Rb transport were used. Thus, 10^{-4} M ouabain, 10^{-5} M bumetanide and 10 μM $GdCl_3$, respectively, were added as inhibitors of Na/K pump, Na-K-2Cl cotransport and channel fluxes. To eliminate Rb fluxes through channels, 2 mM Ba, a broad unspecific inhibitor of channels, and 1 mM tetraethyl ammonium (TEA), a blocker of the BK channel, (White, Darkow & Flavo Lang, 1995; Darkow, Lu & White, 1997) were added. The difference between the Rb fluxes in Cl and sulfamate was calculated as the Cl-dependent Rb influx or K-Cl COT. Details of Rb influx assay are reported elsewhere (Adragna et al., 2000).

Statistical Analysis

Unless otherwise indicated, a minimum of three independent experiments or studies was carried out, where n represents the number of independent determinations per condition (usually 3 to 6) and per experiment. Statistical significance between two groups was evaluated by Student's t-test for paired or unpaired data. A probability of less than 0.05 was considered statistically significant. For densitometrical analysis, multiple intergroup differences in each experiment were determined by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test. A $p < 0.05$ was used as the criterion of statistical significance. Except where indicated, all values were obtained from three independent experiments in which at least triplicate samples were assayed.

Results

Effect of NEM on PKG− and PKG+ Cells

Although the mechanism by which NEM activates K-Cl COT in different tissues is not clearly understood, this drug is widely used as a positive control for the presence or responsiveness of the K-Cl COT system. Thus, we used NEM to determine if the behavior of the system in the new cellular model of PKG− and PKG+ cells was as reported for the cells and tissues tested so far (Lauf et al., 1992; Lauf & Adragna, 2000). Figure 1 shows Rb influx in Cl-containing (Cl) and Cl-free medium (sulfamate, *Sulf*, replacement) and the Cl-dependent component (*K-Cl COT*), determined as the difference between Rb influx in Cl and sulfamate medium, in control and NEM-treated PKG− cells. In control cells, removal of Cl decreased Rb influx by about 50% and in NEM-treated cells, by 70%. Treatment with NEM increased the flux in Cl by about 3-fold and in sulfamate by 1.5-fold, whereas the NEM-induced activation of K-Cl COT was about 5-fold. A 0.5 mM NEM concentration was chosen because we recently reported that the drug activates K-Cl COT in VSMCs at such a concentration (Adragna et al., 2000).

A similar experiment as the one described in Fig. 1 was done for PKG+ cells (Fig. 2). Here, as observed for PKG− cells, removal of Cl from the medium decreased Rb influx by 68% in control and by 75% in NEM-treated cells. In contrast to the be-

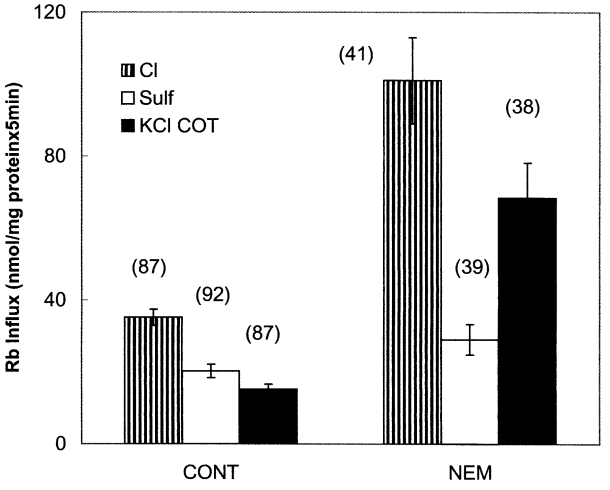

Fig. 1. Effect of N-ethylmaleimide (NEM) on Rb influx in vector-only transfected (PKG−) vascular smooth muscle cells (VSMCs). Culture of cells and determination of Rb influx were as described in Materials and Methods. After the initial wash, cells were preincubated in isotonic buffer saline salt (BSS, 300 mOsM, *see* Materials and Methods), for 15 min without (*CONT*) or with NEM (0.5 mM). Rb uptake was measured for 5 min at 37°C in isotonic medium and in the absence of NEM. Data represent mean ± SEM. The numbers in parentheses are the individual determinations from different experiments pooled together. Hatched bar, Cl; white bar, sulfamate; black bar, Cl-dependent Rb influx or K-Cl COT. Cells were at passages 7–15.

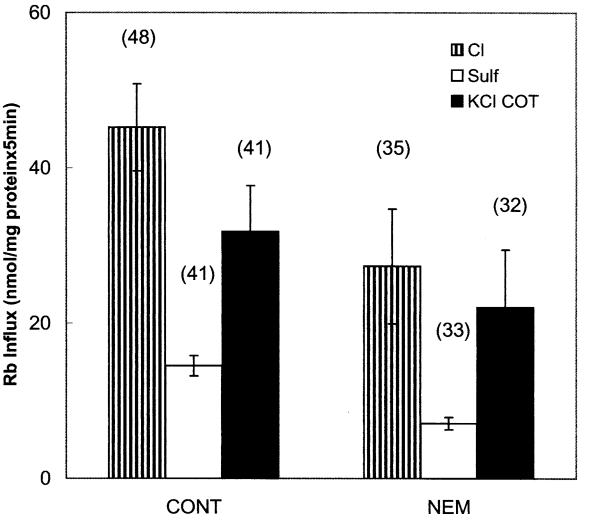

Fig. 2. Effect of NEM on Rb influx in protein kinase G (PKG) Iα catalytic domain-transfected (PKG+) VSMCs. *See* legend to Fig. 1 for culture of cells, determination of Rb influx and symbols. Cells were at passages 9–18.

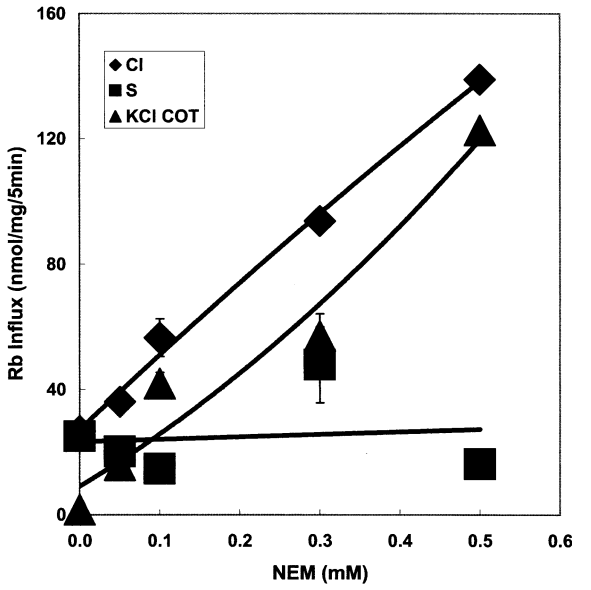

Fig. 3. Concentration-response relationship for NEM in PKG− VSMCs. Culture of cells and determination of Rb influx were as described in Materials and Methods. After the initial wash, cells were preincubated in isotonic BSS for 15 min at different concentrations of NEM (0–0.5 mM). Rb uptake was measured for 5 min at 37°C in isotonic medium and in the absence of NEM in Cl (*diamonds*) and sulfamate (*squares*), and K-Cl COT (*triangles*) was calculated from the difference between the two measurments. Data represent the mean of triplicate samples ± SD. One of three experiments is shown. Cells were at passage 13.

havior of PKG− cells, in PKG+ cells, treatment with NEM decreased the flux in Cl to almost half of the control ($p < 0.00001$) and in sulfamate to almost a third ($p < 0.0001$). K-Cl COT was slightly decreased, although not statistically significant with respect to the control, in NEM-treated cells. Furthermore, the baseline K-Cl COT in PKG+ cells was higher than in PKG− cells ($p < 0.01$).

The conclusion of these experiments is that presence of the PKG I catalytic domain in VSMCs affects the activation of K-Cl COT by NEM. This result could be explained by either a resistance of PKG+ cells to the drug or by a change in the dose-response to NEM or both. To test for the second possibility, PKG− and PKG+ cells were treated with different concentrations of NEM (0–0.5 mM). The resulting Rb influxes in Cl and sulfamate, and the Rb-Cl component are shown in Figs. 3 and 4.

In PKG− cells, Rb-Cl influx increased almost linearly as a function of the NEM concentration in the range of 0–0.5 mM (Fig. 3). Higher concentrations were not tested since the cells detached at these concentrations. Interestingly, in PKG+ cells, NEM activated K-Cl COT by twofold at about 0.05 mM and inhibited this flux by 56% at 0.5 mM (Fig. 4). The maximum NEM activating concentration for Cl and Rb-Cl influx was at 0.05 mM, whereas for sulfamate it was at 0.1 mM, suggesting that in sulfamate, NEM activates Cl-independent Rb/K pathways, different from K-Cl COT. Identical results were found in three experiments done with low-passage (1–3) normal VSMCs containing endogenous PKG (*results not shown*).

These results indicate, on one hand, that transfection of the catalytic domain did not modify the

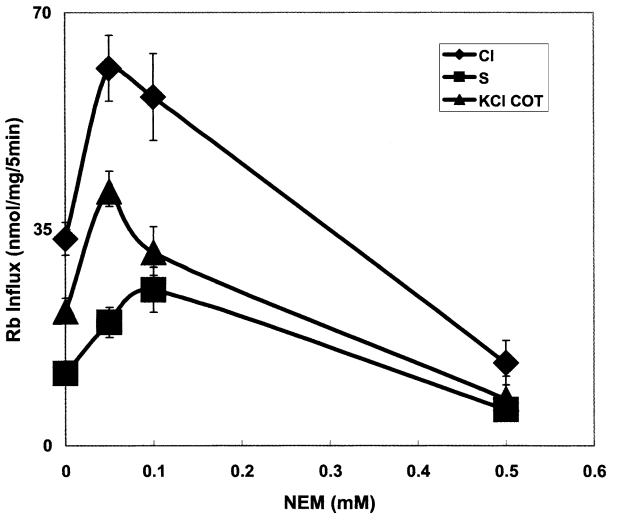

Fig. 4. Concentration-response relationship for NEM in PKG+ VSMCs. Culture of cells, determination of Rb influx and description of figure as described in legend to Fig. 5. Data represent mean ± SEM, $n = 8$ independent experiments each done in triplicate. Cells were at passages 11–18.

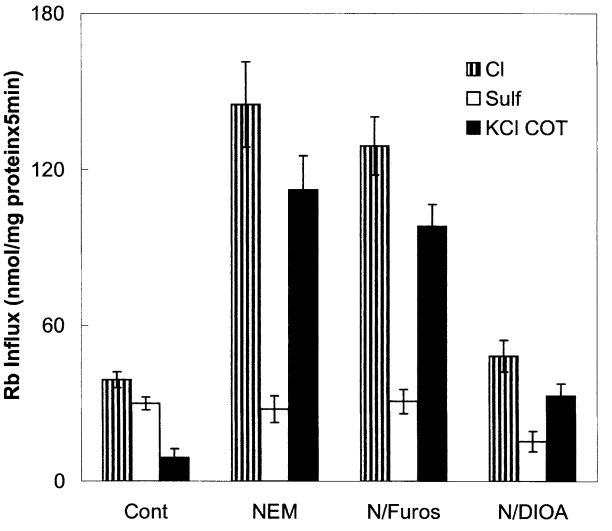

Fig. 5. Effect of NEM, furosemide and [(dihydroindenyl) oxy] alkanoic acid (DIOA) on Rb influx in PKG− VSMCs. Culture of cells and determination of Rb influx were as described in Materials and Methods. After the initial wash, cells were preincubated in isotonic buffer saline salt (BSS, 300 mOsM, *see* Materials and Methods), for 15 min without (CONT) or with NEM (0.5 mM). Rb uptake was measured for 5 min at 37°C in isotonic medium in cells preincubated without NEM (CONT) or with NEM. NEM-treated cells were fluxed either in the presence of 1 mM furosemide (N/Furos) or 300 μM DIOA (N/DIOA). Data represent mean ± SEM. The number of individual determinations from different experiments pooled together varied between 12–17. *Hatched bar*, Cl; *white bar*, sulfamate; *black bar*, Cl-dependent Rb influx or K-Cl COT. Cells were at passages 10–15.

behavior of the transfected cells with respect to non-transfected normal cells, and, on the other hand, that the catalytic domain alone was sufficient to confer an identical response profile to NEM in PKG+ and normal cells. The profile for NEM activation (Figs. 3 and 4) was different in PKG− and PKG+ cells. In PKG+ and normal cells, NEM produced a biphasic response in the range of concentrations used and behaved as a more potent activator.

Effect of Inhibitors of K-Cl COT in PKG− and PKG+ Cells

Two inhibitors of K-Cl COT were chosen, furosemide and DIOA. Furosemide was previously shown not to inhibit K-Cl COT in rat VSMCs (Orlov et al., 1992; Adragna et al., 2000) and, therefore, was selected to indicate whether the vector-transfected (PKG−) cells behaved like the normal non-transfected cells. DIOA was chosen as the most potent inhibitor of K-Cl COT described so far (Lauf et al., 1992; Orlov et al., 1992; Adragna et al., 2000).

Figure 5 shows Rb influx in Cl, sulfamate and Rb-Cl COT in PKG− cells, in control and after treatment with either NEM (0.5 mM) alone, NEM + furosemide (1 mM) or NEM + DIOA (300 μM). Here again, a concentration of 0.5 mM NEM was selected to allow comparison with previous studies done both in VSMCs (Adragna et al., 2000) and in other tissues (Lauf et al., 1992; Lauf & Adragna, 1996). NEM activated the flux in Cl by 4-fold and Rb-Cl COT by 12-fold. No effect was observed in sulfamate. As reported earlier for normal VSMCs (Orlov et al., 1992), in NEM-treated PKG− cells, furosemide slightly decreased (~10%) Rb influx in Cl, as well as Rb-Cl COT, and had no effect in sulfamate. DIOA inhibited the three flux components between 50 to 70% compared to cells treated with NEM alone. These results indicate that the profile of activation and inhibition of K-Cl COT in PKG− cells resembled that of normal VSMCs and that transfection with the vector alone did not induce per se a different behavior.

A similar experiment to that described in Fig. 5 was done for PKG+ cells (Fig. 6). In contrast to the observed results in Fig. 5 and in agreement with those of Fig. 2, PKG+ cells did not respond to NEM stimulation. Note that the baseline K-Cl COT in Fig. 6 was larger than the difference between the average fluxes in Cl and sulfamate, because it represents the mean of individual determinations (*see* figure legend for further details). In spite of the larger dispersion in this set of experiments, the data show that furosemide induced a slight (20 to 30%) but statistically not significant activation in Cl and K-Cl COT and, like in PKG− cells, had no effect in sulfamate. DIOA had no statistically significant effect on either Rb influx in Cl or on K-Cl COT. Although DIOA increased the flux in sulfamate by twofold with respect to that in NEM alone, the effect was not

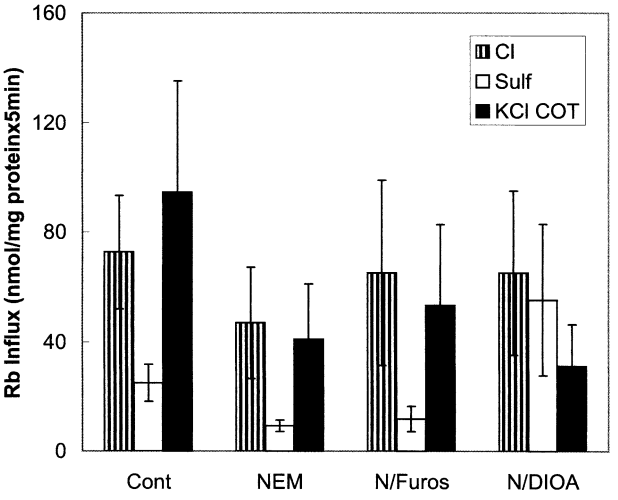

Fig. 6. Effect of NEM, furosemide and DIOA on Rb influx in PKG+ VSMCs. Culture of cells, determination of Rb influx and description of figure as in legend to Fig. 5. The number of individual determinations from different experiments pooled together varied between 5–15. Cells were at passages 13–17. Note that the RbCl influx (*black bar*) in the control (*Cont*) is larger than the difference between the average flux in Cl (*hatched bar*) and that in sulfamate (*white bar*). This apparent discrepancy results from the fact that RbCl influx was calculated for each pair of individual determinations and that in some cases, when either the values in Cl or in sulfamate were missing, the difference could not be calculated.

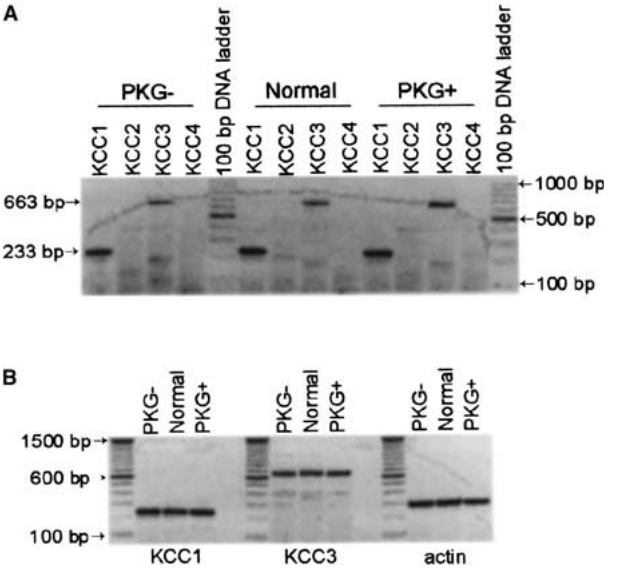

Fig. 7. KCC mRNAs expression in rat VSMCs (normal, PKG− and PKG+). Representative 2% agarose gel electrophoresis stained with ethidium bromide. Cells were cultured as described in Materials and Methods and RT-PCR was performed using total RNA from the following sources: (*A*) Normal, endogenous PKG−depleted vector-only (PKG−)-, and PKG-catalytic domain-transfected (PKG+) VSMCs. All KCC isoforms (1–4) expression was tested using specific sets of primers designed to amplify 233 bp (KCC1), 399 bp (KCC2), 663 bp (KCC3) or 561 bp (KCC4) products. (*B*) Semi quantitative RT-PCR analysis for KCC1 and KCC3 mRNAs was performed, using 1.0 μg total RNA from VSMCs under the conditions described in Materials and Methods.

statistically significant due to the large dispersion in the data. Compared to PKG− cells, the basal K-Cl COT in PKG+ cells was 9-fold higher, but due to the large dispersion in the data for this set of experiments, the results were of borderline statistical significance ($p = 0.09$). Taken together, the results in Figs. 5 and 6 indicate that, whereas PKG− cells behave as normal non-transfected VSMCs at passages higher than 5 (Orlov et al., 1992; Adragna et al., 2000), transfection of the PKG Iα catalytic domain alters the profile of NEM activation of K-Cl COT in PKG+ cells. The mechanisms by which this change may occur are not clear at this point, however, the results do suggest a role for PKG in K-Cl COT regulation in VSMCs.

KCC mRNA Expression in Normal, PKG− and PKG+ Cells

Recently, we reported that serum-deprived VSMCs express at least two KCC isoforms, KCC1 and KCC3, where KCC1 is the most abundant isoform (Di Fulvio et al., 2001) and it was shown that PKG is capable of inducing gene expression in a cGMP-dependent manner, an effect abolished by the selective PKG I inhibitor KT5823 (Di Fulvio et al., 2001). PKG regulation of gene expression remains largely unknown, although some evidence indicates that PKG I may be involved in cAMP-dependent transcription and that the regulation of gene transcription by PKG involves phosphorylation of uncharacterized cytoplasmic protein(s) (Collins & Uhler, 1999).

To assess whether PKG is involved in KCC mRNA expression in serum-fed cells, we determined KCC1/KCC3 mRNA levels in PKG−, normal (containing the endogenous PKG protein, passages 1–3) and PKG+ VSMCs. Figure 7*A* shows the expression pattern of the different KCC isoforms in PKG−, normal and PKG+ serum-fed VSMCs. Simple RT-PCR reactions were performed with KCC1-KCC4-specific primers as described in Materials and Methods. In the three types of cells, only KCC1 and KCC3 mRNA of the expected sizes were detected (Fig. 7*A*). Furthermore, in all the cells, semiquantitative RT-PCR revealed that KCC1 mRNA expression was more abundant than KCC3, both relative to actin expression (Fig. 7*B*). Although PKG− cells were deficient in PKG, normal cells contained the endogenous protein and PKG+ cells possessed only the catalytic domain, KCC mRNA expression was the same in the three types of cells. In contrast, PKG− and PKG+ cells did show differences in baseline K-Cl COT activity and in their response profile to treatment with NEM (Figures 1–6). These results, in conjunction with those in Fig. 7, point to an effect of PKG on K-Cl COT regulation, which is independent of KCC1/KCC3 gene expression.

Discussion

The finding of an activation of K-Cl COT by NO donors and its inhibition by the selective PKG inhibitor KT5823 led us to postulate a potential role for PKG in K-Cl COT regulation (Adragna et al., 1998, 1999; Adragna & Lauf, 1998; Adragna et al., 2000, 2001; Di Fulvio et al., 2001). Thus, PKG− and PKG+ VSMCs were selected as a model to test this hypothesis. In the present study, we provide evidence that K-Cl COT activation by NEM (0.5 mM) and inhibition by furosemide and DIOA in PKG− cells (Figs. 1 and 3) resembled that observed in several tissues, including non-transfected (normal) VSMCs at passages higher than 5 (Lauf et al., 1992; Orlov et al., 1992; Adragna et al., 2000; Lauf & Adragna, 2000), whereas PKG+ cells showed no activation by NEM at this concentration (Figs. 2, 4 and 6). The differences in baseline activation of K-Cl COT and response to NEM, furosemide and DIOA treatment in PKG+ compared to PKG− cells appears to be independent of cGMP binding to PKG, since PKG+ cells only contain the catalytic domain of the enzyme but not the nucleotide-binding site (Boerth & Lincoln, 1994). Furthermore, PKG+ cells and normal cells at early passages (1–3) that contain endogenous PKG showed the same activation profile for NEM (Fig. 4 and results not shown). Additional differences between PKG− and PKG+ cells are the inhibition ($p = 0.001$) and the 3-fold but not statistically significant activation, respectively, induced by DIOA on Rb influx in sulfamate (Figs. 5 and 6). DIOA has recently been shown by us in VSMCs (Anfinogenova et al., 2001) and by Gillen and Forbush in HEK-293 cells (Gillen & Forbush III, 1999) to act on other pathways besides K-Cl COT. In particular, our recent study (Anfinogenova et al., 2001) shows that DIOA can be toxic to VSMCs at concentrations lower than 50 μM, which is normally used for the drug. In the present study, we used 300 μM but the cells did not show signs of toxicity. The major difference with our previous study is that the cells were incubated with DIOA for a total of 25 min, whereas in the present study, the drug was present for only 5 min.

NEM was more potent to activate PKG+ than PKG− cells (Figs. 3 and 4). The half maximal activating concentration (AC_{50}) for NEM activation in various tissues and species is 0.2 mM (Lauf & Adragna, 2000), whereas in PKG+ cells, the maximum activating concentration was found to be 0.05 mM with an AC_{50} about 10-fold lower. The reason for this increased potency is not clear at this point. As mentioned above, although NEM is widely used not only as a thiol reagent but also as an activator of K-Cl COT, its mechanism of action on the system is still unclear. Activation of K-Cl COT by NEM is believed to occur by direct or indirect inhibition of a putative serine/threonine protein kinase (Lauf et al., 1992; Lauf & Adragna, 2000), probably the putative volume-sensitive kinase (Jennings & al-Rohil, 1990; Jennings, 1999). In fact, in rabbit erythrocytes, the rate-limiting inactivation of K-Cl COT is strongly affected by cell volume, supporting although not proving the idea that NEM inhibits either directly or indirectly this kinase (Jennings, 1999). This interesting hypothesis could explain some of our findings in PKG− and PKG+ VSMCs as described below.

Our hypothesis proposes that PKG regulates K-Cl COT. Figures 1 and 2 show that the basal activity of K-Cl COT was higher in PKG+ than in PKG− cells. This suggests that PKG activates K-Cl COT. Treatment of the cells with 0.5 mM NEM induced K-Cl COT activation in PKG− and inhibition in PKG+ cells (Figs. 3 and 4). The activation in PKG− cells (Fig. 3) could be explained by inhibition of the putative volume-sensitive protein kinase (Jennings, 1999), since PKG− cells lack PKG activity. Inhibition of PKG+ cells by 0.5 mM NEM could be explained by a biphasic effect combined with a higher potency of the drug in these cells (Fig. 4). The inhibitory effect of NEM on K-Cl COT supports our previous findings in sheep red blood cells (Lauf & Adragna, 1995). What is difficult to explain at this point is why less NEM is required to maximally activate K-Cl COT in cells containing the catalytic domain of PKG (PKG+ cells).

NEM has a multiplicity of effects, which are dependent on the species, tissue, cell type, drug concentration, type of agonists and antagonists and other factors. Of significant interest to us is the inhibition by NEM of the catalytic activity of cGMP-dependent protein kinase isolated from silkworm pupal fat body (Rochette-Egly & Castagna, 1978). If NEM also inhibits the catalytic activity in PKG+ VSMCs and if PKG activates K-Cl COT as it appears, one should find the inhibition of K-Cl COT by 0.5 mM NEM observed in Fig. 4 for these cells. However, this possibility can only explain the results observed at 0.5 mM NEM but not the activation seen at 0.05 mM (Fig. 6). Perhaps, as reported for G protein-dependent signaling pathways in mouse atria (Foucart, Murphy & Majewski, 1990), inhibition of PKG by NEM in VSMCs may also depend on the concentration of the drug. Likewise, it is unclear if PKG has any effect on the putative volume-sensitive kinase (Jennings, 1999).

As reported elsewhere (Di Fulvio et al., 2001), KCC1 and KCC3 mRNA was highly expressed in rat normal (passage 1–3), PKG−, and PKG+ VSMCs, and KCC1 mRNA was more abundant than KCC3 mRNA (Fig. 7 and Di Fulvio et al., 2001). The major difference between the present studies and those reported elsewhere (Di Fulvio et al., 2001) is that here, the cells were not serum-deprived for 24 hr. Thus,

presence or removal of serum for 24 hr appears to affect neither the expression pattern nor the relative abundance of KCC mRNA isoforms in VSMCs (Fig. 7 and Di Fulvio et al., 2001), suggesting no major involvement of serum or PKG in the maintenance of basal KCC mRNA expression in these cells.

However, transfection of the cGMP-independent PKG Iα catalytic domain did change the activation patterns of K-Cl COT in VSMCs (Figs. 1-6), suggesting some role for PKG in protein phosphorylation along the regulatory cascade (Di Fulvio et al., 2001). It is important to stress, as discussed above (Fig. 7 and Di Fulvio et al., 2001), that PKG doesn't affect the basal expression of KCC mRNA but it does appear to be involved in the acute regulation of KCC mRNA expression by NO donors and cGMP (Di Fulvio et al., 2001). Whatever the mechanism, direct regulation of K-Cl COT by PKG appears to be a plausible mechanism, although elucidation of the actual mechanism awaits further investigation.

In conclusion, our results suggest that PKG is involved in the higher baseline K-Cl COT activity in PKG+ with respect to PKG− VSMCs, and also in the different profile for NEM activation at 0.5 mM NEM. Since both cell types express comparable KCC1/KCC3 mRNA levels, PKG appears to be involved at the post-transcriptional level in K-C1 COT activation.

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