$[Ca^{2+}]_i$ determines the effects of protein kinases A and C on activity of rat renal Na^+, K^+-ATPase

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1. It is well established that the activity of Na^+,K^+-ATPase (NKA) is regulated by protein kinases A (PKA) and C (PKC), but results on their effects have been conflicting. The aim of this study was to examine if this is ascribed to the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$).

2. Rat renal NKA was stably expressed in COS cells (green monkey kidney cells). Increases in $[Ca^{2+}]_i$ were achieved with the Ca^{2+} ionophore A23187 and verified by direct measurements of $[Ca^{2+}]_i$ using fura-2 AM as an indicator. The activity of NKA was measured as ouabain-sensitive $^{86}Rb^+$ uptake and the state of phosphorylation of NKA was monitored with two site-directed phosphorylation state-specific antibodies.

3. Activation of PKA with forskolin decreased NKA activity by $45{\cdot}5 \pm 8{\cdot}9\%$ at low $[Ca^{2+}]_i$ (120 nM) and increased it by $40{\cdot}5 \pm 6{\cdot}4\%$ at high $[Ca^{2+}]_i$ (420 nM). The change in NKA activity by forskolin correlated with the level of increase in $[Ca^{2+}]_i$.

4. The effect of 1-oleoyl-2-acetoyl-*sn*-glycerol (OAG), a specific PKC activator, on the activity of NKA was also Ca^{2+} dependent, being inhibitory when $[Ca^{2+}]_i$ was low ($29{\cdot}3 \pm 3{\cdot}6\%$ decrease at 120 nM Ca^{2+}) and stimulatory when $[Ca^{2+}]_i$ was high ($36{\cdot}6 \pm 10{\cdot}1\%$ increase at 420 nM Ca^{2+}).

5. The α subunit of NKA was phosphorylated under both low and high $[Ca^{2+}]_i$ conditions upon PKA or PKC activation. PKA phosphorylates Ser943. PKC phosphorylates Ser23.

6. To see if the observed effects on NKA activity are secondary to changes in Na^+ entry, we measured NKA hydrolytic activity using permeabilized membranes isolated from cells under controlled Na^+ conditions. A decreased activity at low $[Ca^{2+}]_i$ and no change in activity at high $[Ca^{2+}]_i$ were observed following forskolin or OAG treatment.

7. Purified NKA from rat renal cortex was phosphorylated and inhibited by PKC. This phosphorylation-associated inhibition of NKA was neither affected by Ca^{2+} nor by calmodulin, tested alone or together.

8. We conclude that effect of PKA/PKC on NKA activity is dependent on $[Ca^{2+}]_i$. This Ca^{2+} dependence may provide an explanation for the diversity of responses of NKA to activation of either PKA or PKC.

Na^+,K^+-ATPase (NKA) is an ubiquitous membrane bound protein that transports Na^+ and K^+ across the plasma membrane against their electrochemical gradients (Skou & Esmann, 1992). It plays a pivotal role for many basic cellular functions such as active transport of certain solutes, regulation of cell volume, and restoration of the membrane potential. An altered enzyme activity occurs in a number of clinical disorders, such as hypertension and diabetes (for a review see Laski & Kurtzman, 1996). Therefore, elucidating the regulatory mechanisms that underlie the control of NKA activity has become an important issue.

There is overwhelming evidence that the activity of NKA can be modulated by first messengers as well as by signalling pathways that involve activation of cAMP-dependent protein kinase (PKA) and/or protein kinase C (PKC) (for a review see Ewart & Klip, 1995). It is well established that NKA from several species can be phosphorylated by PKA (Bertorello *et al.* 1991; Fisone *et al.* 1994; Lopina *et al.* 1995) and that rat NKA can be phosphorylated by PKC (Bertorello *et al.* 1991; Feschenko & Sweadner, 1994; Logvinenko *et al.* 1996). Phosphorylation by PKA and PKC has been demonstrated both *in vitro* and

in vivo (Middleton *et al.* 1993; Beguin *et al.* 1994; Fisone *et al.* 1995; Carranza *et al.* 1996*a*; Feschenko & Sweadner, 1997; Cheng *et al.* 1997*b*,*d*; Li *et al.* 1998). Phosphorylation sites for both PKA (Beguin *et al.* 1994; Feschenko & Sweadner, 1994; Fisone *et al.* 1994) and PKC (Beguin *et al.* 1994; Feschenko & Sweadner, 1995; Logvinenko *et al.* 1996) have been identified.

However, studies on the functional effects of PKA/PKC phosphorylation have led to contradictory results. Studies on microsomal NKA preparations from various tissues and species (Limas *et al.* 1973; Tria *et al.* 1974; Braughler & Corder, 1978; Lingham & Sen, 1982; Tung *et al.* 1990; Bertorello *et al.* 1991; Lopina *et al.* 1995) have revealed an inhibitory effect of cAMP or PKA on NKA. Incubation of HeLa cells (Middleton *et al.* 1990) or mouse LTK$^-$ cells (Horiuchi *et al.* 1993) with cAMP is accompanied by a decrease in NKA enzymatic activity. In contrast, injection or superfusion of *Xenopus* oocytes with cAMP has a clear stimulatory effect on the pump current (Vasilets & Schwarz, 1992). In rat renal proximal tubules, activation of PKA has been reported to cause both a decrease (Hussian *et al.* 1997; Holtbäck *et al.* 1998) and an increase (Carranza *et al.* 1996*b*) in pump activity. Similar conflicting results have been reported for PKC activation, including inhibitory effects (Shahedi *et al.* 1992; Beron *et al.* 1997; Singh & Linas, 1997), no change (Feschenko & Sweadner, 1997) and stimulatory effects (Mito & Delamere, 1993; Sampson *et al.* 1994). Activation of PKC in isolated rat renal proximal tubules (Bertorello & Aperia, 1989; Satoh *et al.* 1993; Feraille *et al.* 1995) as well as in OK (opossum kidney) cells (Middleton *et al.* 1993; Pedemonte *et al.* 1997) has also been reported to cause both inhibitory and stimulatory effects.

Since the discrepancies in results cannot be explained only by species differences, it is likely that differences in experimental conditions have also played a role. One such condition may be the level of intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$), which is highly dependent on buffer conditions and on the preparation of tissues and cells. There is evidence that Ca^{2+} can modulate the activity of NKA (for review see Yingst, 1988). Studies performed on isolated guinea-pig ventricular myocytes have indicated that the effect of the β-adrenergic agonist isoproterenol (isoprenaline) on the NKA current is $[Ca^{2+}]_i$ dependent (Gao *et al.* 1992). Here we show that $[Ca^{2+}]_i$ does indeed modulate the response of rat renal NKA to PKA and PKC activation. Most of the protocols in this study were performed on green monky kidney COS cells stably expressing rat renal NKA. We have previously shown that in this cell system rat NKA is inhibited upon PKA or PKC activation (Belusa *et al.* 1997; Cheng *et al.* 1997*b*,*d*). We now show that the inhibitory effects of PKA/PKC phosphorylation is reversed by increases in $[Ca^{2+}]_i$. A portion of this work has been presented at the 14th International Congress of Nephrology '97, Sydney, Australia (Cheng *et al.* 1997*c*) and at the 30th American Society of Nephrology Annual meeting '97, San Antonio, TX, USA (Cheng *et al.* 1997*a*).

METHODS

Purification of rat renal NKA

NKA was purified from rat renal cortex as described (Jorgensen, 1974). Forty-day-old Sprague–Dawley rats were deeply anaesthetized with pentobarbital (80 mg (kg body weight)$^{-1}$ I.P.). The aorta was cut to empty the organs of blood and the kidneys rapidly removed. The preparation was analysed by SDS–PAGE, stained with Coomassie Brilliant Blue. Densitometric analysis of the stained gels indicated that NKA α subunit constituted 55–60% of total protein. The specific activity of NKA in the preparation used was calculated as ~700 μmol of ATP hydrolysed (mg protein)$^{-1}$ h^{-1}. The ouabain (5 mM)-insensitive ATPase activity was less than 3% of total ATPase activity.

Phosphorylation of purified NKA by PKC *in vitro*

Phosphorylation of purified NKA was carried out in a reaction volume of 50 μl containing: 50 mM Hepes (pH 7·5), 10 mM $MgCl_2$, 1·2 mM $CaCl_2$, 1·25 μg purified NKA, and 4 μU PKC (final concentration ~80 ng ml^{-1}). In control samples, PKC was omitted and an equal volume of bovine serum albumin (BSA) (final concentration 80 ng ml^{-1}) dissolved in PKC storage buffer (50% glycerol (v/v), 0·02% Tween 20 (w/v), 20 mM Tris-HCl, 0·5 mM EGTA, 5 mM dithioerythritol, pH 7·5) was added to the reaction mix. The reaction was initiated by the addition of ATP to a final concentration of 0·1 mM. In some experiments tracer amounts of [γ-^{32}P]ATP were included in the reaction mix to confirm that NKA was phosphorylated. The incubation proceeded at 25 °C for 1 h and was stopped by cooling on ice. The phosphorylation of NKA was verified by detecting the radioactivity of ^{32}P incorporated into the NKA α subunit as described (Fisone *et al.* 1994) or by detecting the changes in the immunoreactivity to a phosphorylation state-specific antibody (Feschenko & Sweadner, 1997) of NKA that had been subjected to SDS–PAGE and transferred to nitrocellulose membranes.

Expression of NKA α subunit in COS cells

The entire cDNA coding for wild-type rat NKA α1 was excised from a Bluescript vector and subcloned into the eukaryotic expression vector pXM as previously described (Cheng *et al.* 1997*b*). The vector pXM contains the adenovirus major late promoter and the SV40 origin, early gene enhancer and polyadenylation sequence. To obtain cell lines with the NKA cDNA stably integrated into chromosomes, plasmid DNA was linearized with *Nde*I and transfected into COS-7 cells using the calcium-phosphate–DNA precipitation method. We used ouabain sensitivity to select those COS cells in which the cDNA encoding the rat α1 subunit had been stably transfected (Cheng *et al.* 1997*b*). Due to a difference in ouabain binding affinity between monkey and rat NKA, monkey cells expressing the rat NKA survive concentrations of ouabain that kill wild-type monkey cells (COS-7 cells). Cells were cultured in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal calf serum (FCS). Sixty hours following cell transfection, ouabain was added to the medium to a final concentration of 10^{-5} M. After about 10 days, hundreds of individual ouabain-resistant colonies appeared. These colonies were pooled and propagated. The method has been described in detail previously (Cheng *et al.* 1997*b*). In the surviving cells, the transfected NKA represents approximately 50–60% of total NKA.

Cell culture

Cells were grown at 37 °C, 5% CO_2, and 95% humidified air on 90 mm culture dishes or 6-well culture plates in DME medium supplemented with 10% fetal calf serum (FCS) and 1% Pen-Strep (1% penicillin–streptomycin). A low concentration of ouabain

(10^{-5} M) was present in the medium to block endogenous NKA activity (Cheng *et al.* 1997*b*). The number of cells was selected to seed on the culture plates so that they would reach confluency in 4 or 5 days. Media were changed 16 h before the cells were used for experiments.

Increases in intracellular Ca^{2+}

To increase the level of $[Ca^{2+}]_i$, cells were preincubated with varying amounts of Ca^{2+} ionophore A23187 (from 10^{-8} to 10^{-5} M) to obtain $[Ca^{2+}]_i$ ranging between 120 and 420 nM. The calcium concentration in incubation solution was 1 mM. The levels of $[Ca^{2+}]_i$ were determined by direct measurements of intracellular Ca^{2+} as described below. In the continuous presence of A23187, an increase in $[Ca^{2+}]_i$ occurred within 1 min following the addition of the drug, which reached a plateau after about 5 min that lasted for at least 15 min (Fig. 1). Thus, to test the effect of Ca^{2+}, cells were preincubated with the Ca^{2+} ionophore 5 min before PKA or PKC was activated. The kinetics of the $[Ca^{2+}]_i$ change was similar with different concentrations of A23187 used.

Activation of PKA and PKC

To activate PKA, confluent cells were incubated with forskolin (10^{-5} M), used in combination with the phosphodiesterase inhibitor IBMX (5×10^{-4} M). To activate PKC, OAG (10^{-8} M), the diacyglycerol analogue, was used. Unless otherwise specified, all the incubations were performed for 15 min at 37 °C in a physiological salt solution (PSS) containing (mM): 110 NaCl, 5 KCl, 1·0 $CaCl_2$, 1·2 $MgCl_2$, 25 $NaHCO_3$, 1·0 NaH_2PO_4, 20 Hepes (pH 7·4 at 37 °C), and 10 glucose. All the cell incubation solutions were prewarmed at 37 °C and equilibrated with 5% CO_2 and 95% O_2 before applied to the cell cultures.

Cell lysate preparation

Following drug treatment, cells were incubated for 15 min on ice in a lysis buffer containing: 130 mM NaCl, 5 mM EDTA, 2 mM EGTA, 20 mM Tris-HCl (pH 8·0), 50 mM NaF, 1% (w/w) Triton X-100, 0·1% (w/w) SDS, 100 μM phenylmethylsulfonyl fluoride (PMSF), 25 mM benzamidine, 20 μg ml^{-1} leupeptin, 20 μg ml^{-1} antipain, 5 μg ml^{-1} pepstatin A, 5 μg ml^{-1} chymostatin, and 0·2% (w/v) BSA. Cells were then scraped and briefly sonicated. Protein concentrations were determined using a kit from Bio-Rad and BSA as a standard. Samples containing equal amounts of protein were subjected to SDS–PAGE and immunoblotting.

Western blot analysis and determination of phosphorylation

Electrophoresis was performed using an 8% polyacrylamide gel. Protein transfer, immunoblotting and phosphorylation determination were performed as previously described (Cheng *et al.* 1997*b*).

Two different site-selective phosphorylation state-specific antibodies were used in this study: monoclonal antibody Mck1, which specifically binds to the Ser23-dephosphorylated, but not the phosphorylated, form of rat NKA α1 subunit (Feschenko & Sweadner, 1997); and polyclonal antibody 471, which selectively detects the Ser943-phosphorylated, but not the dephosphorylated, form of NKA α subunit (Fisone *et al.* 1994). For comparison, the abundance of NKA protein was also examined. This was achieved by using an antibody (McAb 6F, 1:100 dilution) that is not sensitive to changes in phosphorylation (Feschenko & Sweadner, 1997).

Measurement of intracellular Ca^{2+}

Single cell-based $[Ca^{2+}]_i$ measurements were made in a SPEX dual-beam excitation spectrofluometer using fura-2 as an indicator. Cells grown on 25 mm glass coverslips were loaded with a membrane-permeable acetoxymethyl (AM) ester of fura-2 (2 μM) at 37 °C for 1 h under standard culture conditions as described above. The cells were then washed and subsequently superfused with a PSS solution. The Petri dish was placed on the thermostable stage of a fluorescence microscope (Zeiss Inc., Thornwood, NY, USA) equipped with a fluorescence detection and quantification system. Cytosolic free Ca^{2+} concentration was calculated from the ratio of fluorescence detected at 510 nm using excitation wavelengths of 345 and 380 nm. The fluorescence ratio signal was calibrated at the end of each experiment by adding 1 μM ionomycin to equilibrate the cytoplasm with the external PSS solution where the Ca^{2+} concentration was 1 mM. This solution was then replaced with a PSS solution containing 5 mM EGTA. $[Ca^{2+}]_i$ was calculated according to the equation described by Grynkiewicz *et al.* (1985) assuming a K_d of fura-2 for Ca^{2+} of 224 nM. All experimental solutions were kept at 37 °C and equilibrated with 5% CO_2 and 95% O_2.

Measurement of $^{86}Rb^+$ uptake

The transport activity of NKA was determined in intact cells by measuring ouabain-sensitive $^{86}Rb^+$ uptake under linear conditions. The experimental details have been given elsewhere (Cheng *et al.* 1997*b*). Experiments were performed at 37 °C under a humidified atmosphere with 5% CO_2–95% O_2 in the PSS solution. The incubation buffer was prewarmed at 37 °C and equilibrated with 5% CO_2–95% O_2. The uptake of $^{86}Rb^+$ from the medium was calculated on the basis of the specific activity of the incubation medium. Ouabain-sensitive $^{86}Rb^+$ uptake was determined as the difference between $^{86}Rb^+$ uptake in the absence and presence of 5 mM ouabain.

Determination of NKA activity

Isolation and permeabilization of cell membranes and subsequent determination of NKA activity were performed as described (Fisone *et al.* 1994; Cheng *et al.* 1997*b*). In brief, following preincubation of cells with drugs, cell membranes were isolated, resuspended in TME buffer (75 mM Tris (pH 7·5), 12·5 mM $MgCl_2$, 1·5 mM EDTA) and quickly frozen on dry ice and thawed at room temperature to open vesicles formed during the membrane

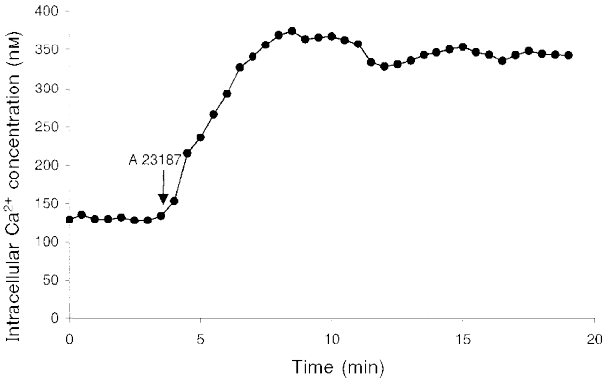

Figure 1. The kinetic change in $[Ca^{2+}]_i$ induced by A23187

$[Ca^{2+}]_i$ was measured using fura-2 AM as an indicator on SPEX dual-beam excitation spectrofluometer as described in Methods. Shown here is an example of changes in $[Ca^{2+}]_i$ induced by A23187, which was added at 10^{-6} M. Similar kinetic changes induced by different concentrations of A23187 were also observed. The data represent mean values obtained from 12 individual cells in one experiment.

preparation. Aliquots of membrane fragments were incubated for 15 min at 37 °C in 100 μl of a solution containing (mM): 20 KCl, 5 $MgCl_2$, 30 Tris-HCl (pH 7·4), 1 EGTA, 3 Tris-ATP, and tracer amounts of [γ-^{32}P]-ATP. NaCl was added to 10 or 70 mM. To maintain a constant osmolarity, choline chloride was also added, so that the sum of NaCl and choline chloride was kept constant at 130 mM. To prevent dephosphorylation of Na^+,K^+-ATPase, the buffer was complemented with the protein phosphatase inhibitors okadaic acid (250 nM) and FK506 (25 nM). An amount of enzyme was selected so that total ATP hydrolysis did not exceed 20 %, and ATP hydrolysis was linear with time. The reaction was stopped by the addition of 700 μl of activated charcoal. The $^{32}P_i$ liberated was determined in the supernatant after centrifugation. For the determination of ouabain-insensitive rat ATPase activity, NaCl and KCl were omitted, and 5 mM ouabain was added. The protein content of cell membranes was determined as described above.

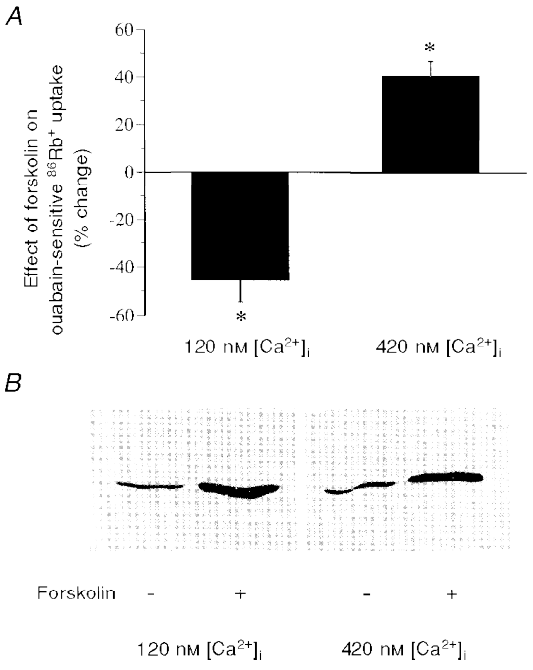

Figure 2. The effects of forskolin and IBMX on the activity and Ser943 phosphorylation of NKA at both low and high $[Ca^{2+}]_i$

Confluent cultures of COS cells expressing rat renal NKA were treated with forskolin (10^{-5} M) and IBMX (5×10^{-4} M) for 15 min at 37 °C. Following drug treatment, both the activity and Ser943 phosphorylation of NKA were assayed. The levels of $[Ca^{2+}]_i$ were measured in a SPEX dual-beam excitation spectrofluometer using fura-2 AM as an indicator. An increase in $[Ca^{2+}]_i$ was achieved by preincubation of cells with the Ca^{2+} ionophore A23187 (10^{-5} M). The $[Ca^{2+}]_i$ values shown here represent the mean values of the $[Ca^{2+}]_i$ from 16–21 individual cells in four separate experiments, which were calculated based on an *in vivo* calibration system (see Methods). *A*, the effect of forskolin and IBMX on the activity of NKA, which was measured as ouabain-sensitive $^{86}Rb^+$ uptake. Values are means ± S.E.M. of 5 separate experiments. The basal NKA activity (in nmol mg^{-1} min^{-1}) was 18·75 ± 2·10 at low $[Ca^{2+}]_i$ and 20·62 ± 2·53 at high $[Ca^{2+}]_i$. *B*, the effect of forskolin and IBMX on the state of Ser943 phosphorylation, which was determined by Western blot analysis with an antibody specifically recognizing the Ser943-phosphorylated, but not the dephosphorylated, form of NKA. A blot representative of 3 experiments is shown. * $P < 0·01$.

The determination of NKA activity in purified enzyme preparations was essentially made using the same protocol as described above except that the reaction was made in a buffer containing (mM): 100 NaCl, 5 KCl, 5 $MgCl_2$, 50 Tris-HCl (pH 7·4 at 37 °C), 3 Tris-ATP, and tracer amounts of [γ-^{32}P]ATP. To study the effect of Ca^{2+} and calmodulin on the NKA activity, Ca^{2+} present in the reaction mix was removed by dilution and centrifugation using a Centricon concentrator (Amicon Inc., Beverly, MA, USA). The concentrated sample was washed twice with ice-cold SH buffer (250 mM sucrose and 30 mM histidine, pH 7·2), and the pellet was resuspended in 50 μl SH buffer for the determination of NKA activity. Control and phosphorylated enzymes were treated identically. The different concentrations of Ca^{2+} in the assay medium were obtained using a combination of $CaCl_2$ and EGTA. The concentrations of free Ca^{2+} in buffers were calculated as described (Bers *et al.* 1994) using a computer program (MAXCHELATOR, Stanford University: http://www.stanford.edu/~cpatton) in which all the substances that could possibly influence the binding of Ca^{2+} to EGTA were taken into account.

Chemicals

Forskolin, IBMX, monensin and EGTA were purchased from Sigma; 1-oleoyl-2-acetoyl-*sn*-glycerol (OAG) was from Avanti Polar Lipids, Inc., Alabaster, AL, USA; the bisindolyl maleimide GF109203x and A23187 were from Calbiochem; fura-2 AM and SBFI were from Molecular Probes. All drugs were stored as stock solutions in DMSO at −20 °C. The final concentration of DMSO in the working solutions was 0·1 % (v/v), which was always added as vehicle to each control solution. Ouabain was purchased from Merck; PKC from rat brain and calmodulin were purchased from Boehringer-Mannheim; ^{86}RbCl was from Amersham International; [γ-^{32}P]-ATP was from New England Nuclear; and DMEM, fetal calf serum (FCS) and 1 % Pen-Strep were from Life Technologies Inc.

Statistics

Values are given as means ± S.E.M. Statistical comparisons between two groups were performed by Student's *t* test. $P < 0·05$ was considered significant.

RESULTS

Effect of forskolin on the activity and Ser943 phosphorylation of NKA at both low and high $[Ca^{2+}]_i$

To assess the possible role of $[Ca^{2+}]_i$ in the regulation of NKA activity by PKA, cells were pretreated with the Ca^{2+} ionophore A23187. An amount of the Ca^{2+} ionophore was selected so that different levels of increase in $[Ca^{2+}]_i$ were produced. The increases in $[Ca^{2+}]_i$ were verified by direct intracellular Ca^{2+} measurement using fura-2 as an indicator. Following A23187 pretreatment, cells were incubated for 15 min at 37 °C with forskolin (10^{-5} M) plus the phosphodiesterase inhibitor IBMX (5×10^{-4} M) to activate PKA. The activity of NKA was measured by $^{86}Rb^+$ uptake and the state of phosphorylation of Ser943, the site of the NKA α subunit phosphorylated by PKA, was assessed. At low $[Ca^{2+}]_i$ (120 ± 14 nM, $n = 21$), activation of PKA by forskolin and IBMX caused a significant decrease (45·5 ± 8·9 %) in ouabain-sensitive $^{86}Rb^+$ uptake (Fig. 2*A*). In contrast, at high $[Ca^{2+}]_i$ (420 ± 32 nM, $n = 16$), forskolin

and IBMX caused a significant increase (40·5 ± 6·4%) in ouabain-sensitive $^{86}Rb^+$ uptake. The basal NKA activity (in nmol mg^{-1} min^{-1}) was 18·75 ± 2·10 at low $[Ca^{2+}]_i$ and 20·62 ± 2·53 at high $[Ca^{2+}]_i$, values which are not statistically different ($P > 0·05$).

The phosphorylation of Ser943 was analysed by Western blot using an antibody that selectively recognizes the Ser943-phosphorylated, but not the dephosphorylated, form of NKA (Fisone *et al.* 1994; Cheng *et al.* 1997*b*) (Fig. 2*B*). For comparison, the abundance of NKA protein was also examined using an antibody that is not sensitive to changes in phosphorylation (Feschenko & Sweadner, 1997). At both low and high $[Ca^{2+}]_i$, activation of PKA by forskolin and IBMX caused a significant increase in phosphorylation of NKA. The percentage increase in immunoreactivity by forskolin and IBMX was 191·3 ± 23·2% at low $[Ca^{2+}]_i$ ($P < 0·01$) and 186·1 ± 34·6% at high $[Ca^{2+}]_i$ ($P < 0·01$). Under both $[Ca^{2+}]_i$ conditions, the abundance of NKA was not significantly affected by forskolin and IBMX treatment. The percentage change in immunoreactivity was 103·3 ± 4·4% at low $[Ca^{2+}]_i$ ($P > 0·05$) and 106·1 ± 4·7% at high $[Ca^{2+}]_i$ ($P > 0·05$).

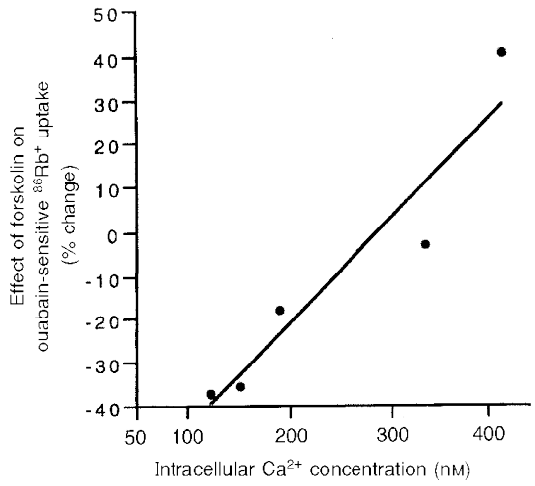

Figure 3. The relationship between the levels of $[Ca^{2+}]_i$ and changes in NKA activity induced by forskolin and IBMX

The percentage changes in NKA activity induced by forskolin (10^{-5} M) and IBMX (5×10^{-4} M) (mean values of 2–4 separate experiments) were plotted as a function of the levels of $[Ca^{2+}]_i$ at which the regulation of NKA activity was performed. The activity of NKA in both control and forskolin- and IBMX-incubated cells was measured as ouabain-sensitive $^{86}Rb^+$ uptake. The different levels of $[Ca^{2+}]_i$ were produced by the application of different concentrations of A23187 and were measured in a SPEX dual-beam excitation spectrofluometer as described in the Methods. The $[Ca^{2+}]_i$ values shown here represent the mean values of the $[Ca^{2+}]_i$ from 16–21 individual cells in 4 separate experiments. These five $[Ca^{2+}]_i$ values are (mean ± S.E.M.) 120 ± 14, 151 ± 15, 188 ± 23, 331 ± 24 and 420 ± 32 nM, which represent the levels of $[Ca^{2+}]_i$ of control cells and cells treated with A23187 at 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M, respectively.

To examine whether there was a quantitative relationship between $[Ca^{2+}]_i$ and the effect of forskolin on NKA activity, we performed a series of experiments in which various amounts of A23187 were used (Fig. 3). Increasing $[Ca^{2+}]_i$ initially attenuated and then reversed the inhibitory effect of forskolin and IBMX on NKA activity in an approximately linear fashion.

Effect of OAG on the activity and Ser23 phosphorylation of NKA at both low and high $[Ca^{2+}]_i$

To assess the possible role of $[Ca^{2+}]_i$ in the regulation of NKA activity by PKC, cells were treated as above with A23187 and the effects of OAG, an activator of the classic and novel isoforms of PKC (Nishizuka, 1984), were measured (Fig. 4*A*). At low $[Ca^{2+}]_i$, activation of PKC by OAG caused a significant decrease (29·3 ± 3·6%) in ouabain-sensitive $^{86}Rb^+$ uptake. In contrast, OAG caused a significant increase (36·6 ± 10·1%) in ouabain-sensitive $^{86}Rb^+$ uptake at high $[Ca^{2+}]_i$.

The effect of OAG on the state of phosphorylation of Ser23, the site phosphorylated by PKC, was measured

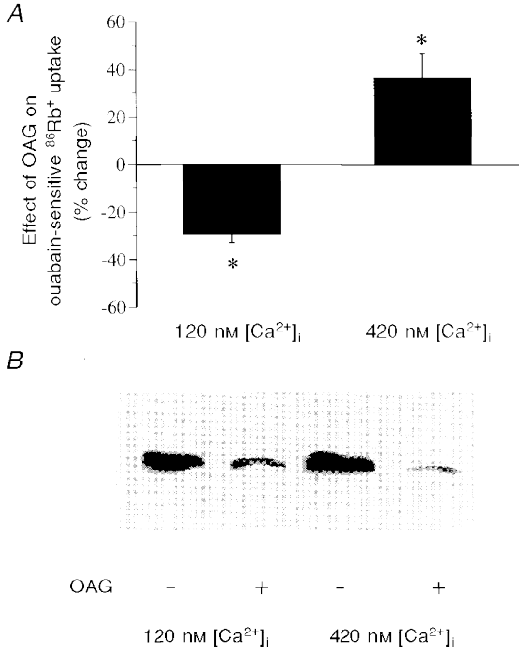

Figure 4. The effects of OAG on the activity and Ser23 phosphorylation of NKA at both low and high $[Ca^{2+}]_i$

Confluent cultures of COS cells expressing rat renal NKA were treated with OAG (10^{-8} M) for 15 min at 37 °C under low and high $[Ca^{2+}]_i$ conditions. Following drug treatment, both the activity and Ser23 phosphorylation of NKA were assayed. *A*, the effect of OAG on the activity of NKA. Values are means ± S.E.M. of 5 separate experiments. The basal NKA activity (in nmol mg^{-1} min^{-1}) was 18·75 ± 2·10 at low $[Ca^{2+}]_i$ and 20·62 ± 2·53 at high $[Ca^{2+}]_i$. *B*, the effect of OAG on the state of Ser23 phosphorylation, which was determined by Western blot analysis with an antibody specifically recognizing the Ser23-dephosphorylated, but not the phosphorylated, form of NKA. A blot representative of 3 experiments is shown. * $P < 0·01$.

Table 1. NKA activity in cell membranes isolated from cells pretreated with forskolin or OAG under low or high $[Ca^{2+}]_i$ conditions

	NKA activity (nmol mg^{-1} h^{-1})	
	10 mM Na^+	70 mM Na^+
Low $[Ca^{2+}]_i$		
Control	2285 ± 195	5211 ± 329
FSK + IBMX	1452 ± 217 *	4228 ± 477 *
OAG	1419 ± 162 *	4044 ± 388 *
High $[Ca^{2+}]_i$		
Control	2132 ± 218	5043 ± 585
FSK + IBMX	2136 ± 376	5588 ± 877
OAG	2529 ± 492	5302 ± 954

COS cells stably expressing rat renal NKA were incubated with forskolin (FSK; 10^{-5} M) and IBMX (5×10^{-4} M) or OAG (10^{-8} M) for 15 min at 37 °C at low (120 nM) or high (420 nM) $[Ca^{2+}]_i$. Following incubation, membranes were isolated and permeabilized, and Na^+, K^+-ATPase activity was measured as ouabain-sensitive ATP hydrolysis in the presence of protein phosphatase inhibitors okadaic acid and FK506 at non-saturating (i.e. 10 mM) and saturating (i.e. 70 mM) levels of Na^+ as described in Methods. Values represent means ± S.E.M. of 7 separate experiments. * Significantly different from control, $P < 0{\cdot}01$, Student's paired t test.

Table 2. Inhibition of the activity of rat renal Na^+, K^+-ATPase by PKC phosphorylation: effects of Ca^{2+} and calmodulin (CaM)

		Na^+,K^+-ATPase activity		
Ca^{2+} (μM)	CaM (μM)	Control (μmol P_i mg^{-1} h^{-1})	PKC phosphorylation (μmol P_i mg^{-1} h^{-1})	% control
0	0	610 ± 7	345 ± 28 *	56·7 ± 5·0
10	0	577 ± 17	327 ± 32 *	56·9 ± 6·6
10	1	621 ± 13	347 ± 31 *	56·2 ± 6·0
1000	0	513 ± 14	297 ± 28 *	58·1 ± 5·8
1000	1	656 ± 38	365 ± 43 *	55·9 ± 7·4

Na^+,K^+-ATPase purified from rat renal cortex was phosphorylated *in vitro* by PKC as described under the Methods. The hydrolytic activity of Na^+,K^+-ATPase was assayed in 100 μl of reaction mixture containing (mM): 100 NaCl, 5 KCl, 5 $MgCl_2$, 50 Tris-HCl (pH 7·4 at 37 °C), 3 Tris-ATP, and tracer amounts of [γ-^{32}P]ATP in the absence or presence of listed additives (i.e. Ca^{2+} and CaM). Values are means ± S.E.M. of 5 separate experiments. * Significantly different from control, $P < 0{\cdot}01$.

(Fig. 4*B*). In this study, the phosphorylation of Ser23 was also monitored by Western blot analysis using a phosphorylation state-specific antibody. Unlike the antibody used for detection of the Ser943 phosphorylation, this antibody selectively reacts with the dephosphorylated, but not the phosphorylated Ser23 of the NKA α subunit (Feschenko & Sweadner, 1997). Under basal conditions, an immunoreactive band corresponding to the α subunit of NKA (molecular mass ~110 kDa; Fig. 4*B*, lanes 1 and 3) was detected in cell lysates. Activation of PKC by OAG significantly decreased the immunoreactive signal at both low and high $[Ca^{2+}]_i$ (Fig. 4*B*, lanes 2 and 4). The percentage decrease in immunoreactivity was 56·7 ± 3·6 % at low $[Ca^{2+}]_i$ ($P < 0{\cdot}01$) and 64·9 ± 7·3 % at high $[Ca^{2+}]_i$ ($P < 0{\cdot}01$). Under both $[Ca^{2+}]_i$ conditions, the abundance of NKA was not significantly affected by OAG treatment. The percentage change in immunoreactivity was 98·2 ± 10·1 % at low $[Ca^{2+}]_i$ ($P > 0{\cdot}05$) and 101·6 ± 8·9 % at high $[Ca^{2+}]_i$ ($P > 0{\cdot}05$).

Role of Na^+ entry in the regulation of the NKA activity by forskolin or OAG

To investigate if the observed inhibition or stimulation of NKA activity induced by forskolin or OAG is secondary to changes in $[Na^+]_i$ brought about by putative changes in Na^+ entry, we measured NKA activity using permeabilized membranes isolated from these cells under controlled Na^+ conditions. The results are summarized in Table 1. In this study, cells were treated with forskolin or OAG as described

above under both low and high $[Ca^{2+}]_i$ conditions. Following drug treatment, cells were lysed and crude cell membranes were prepared in the presence of protein phosphatase inhibitors okadaic acid and FK506. The activity of NKA was assayed as ouabain-sensitive ATP hydrolysis in 10 and 70 mM Na^+, respectively. At low $[Ca^{2+}]_i$, activation of PKA with forskolin and IBMX resulted in a significant decrease in NKA activity, measured under both non-saturating (i.e. 10 mM) and saturating (i.e. 70 mM) Na^+ conditions. The results are consistent with the effects on NKA-dependent Rb^+ fluxes assayed in intact cells. At high $[Ca^{2+}]_i$, no change in NKA activity was observed following PKA activation. Similar changes in NKA activity were also observed for OAG-induced PKC activation (Table 1). The basal activity of NKA was lower at high $[Ca^{2+}]_i$ than at low $[Ca^{2+}]_i$ but the difference was not statistically significant.

Effects of Ca^{2+}–calmodulin on PKC-mediated regulation of purified NKA

To examine whether Ca^{2+} exerts its effect on NKA via a direct interaction with the enzyme, we phosphorylated NKA *in vitro* and compared the phosphorylation-induced changes in NKA activity in the absence or presence of Ca^{2+} and/or its binding protein, calmodulin. The activity of NKA was measured as ouabain-sensitive ATP hydrolysis.

Phosphorylation of NKA by PKC resulted in a significant decrease in NKA activity (Table 2). When the specific PKC inhibitor bisindolyl maleimide GF109203x was present, both phosphorylation and inhibition of NKA were abolished (data not shown). To examine the effect of Ca^{2+}, we added to the assay buffer various concentrations of Ca^{2+} and/or calmodulin. As shown in Table 2, the inhibition of NKA activity by PKC phosphorylation was not affected by Ca^{2+} or by calmodulin, tested alone or together.

DISCUSSION

Regulation of NKA activity by hormones and second messengers has been extensively studied (for a review see Ewart & Klip, 1995). Although it is generally agreed that rapid changes in NKA activity can be achieved by agents that affect the activity of PKA and/or PKC, the direction in which these substances affect NKA varies significantly. The present study shows that the level of $[Ca^{2+}]_i$ is one important determinant in the response of NKA to second messenger activation, and suggests that future studies of the role of PKA/PKC activation in the regulation of NKA activity should be performed under controlled $[Ca^{2+}]_i$ conditions.

Table 3 summarizes some of the most relevant studies on short-term regulation of NKA activity by PKA/PKC. Notably, most of the studies in which an inhibitory effect was observed were performed using Ca^{2+}-free conditions. On the other hand, a stimulatory effect was often observed using conditions where the $[Ca^{2+}]_i$ had been high or might have been rendered high by experimental manipulation. This may, for instance, have been the case when cells were subjected to pretreatment with a K^+-free solution. K^+-free pretreatment will lead to an inhibition of NKA activity and a subsequent increase in intracellular Na^+ ($[Na^+]_i$). The latter might in turn lead to an inhibition of the Na^+–Ca^{2+} exchanger and an increase in $[Ca^{2+}]_i$ (Blaustein, 1993). Another example in which $[Ca^{2+}]_i$ might have been increased is the study in which a high osmolarity solution (400 mosmol kg^{-1}) was used. A decrease in cell volume resulting from the increase in osmolarity of the medium may trigger an increase in $[Ca^{2+}]_i$ (Watson, 1991).

In this study, we employed two different methods to measure the functional effect of NKA, i.e. $^{86}Rb^+$ fluxes which measure the transport activity of NKA under *in vivo* conditions, and ATP hydrolysis which was performed in permeabilized cell preparations under controlled ionic conditions. While the former provides the best evaluation of the contribution of NKA to cation transport of intact cells, the latter allows us to discriminate primary active cation transport (through the pump) from secondary active transport (such as that mediated by changes in Na^+ entry). With these two methods, we found that activation of PKA or PKC led to inhibition of NKA activity at low $[Ca^{2+}]_i$, as reflected by a decrease in ATP hydrolysis and $^{86}Rb^+$ uptake, and stimulation of $^{86}Rb^+$ uptake but no change in ATP hydrolysis at high $[Ca^{2+}]_i$. The results suggest that the PKA/PKC inhibition of NKA at low $[Ca^{2+}]_i$ is direct. The stimulatory effect of forskolin or OAG on ouabain-sensitive Rb^+ uptake at high $[Ca^{2+}]_i$ may be due to a combined effect, i.e. release of PKA/PKC-induced inhibition of NKA by Ca^{2+} modulation and secondary stimulation of NKA by an increase in Na^+ entry. In accordance with this hypothesis, we have found in ongoing studies that activation of PKA by forskolin and IBMX causes an increase in $[Na^+]_i$ under low $[Ca^{2+}]_i$ conditions but no change in $[Na^+]_i$ when high $[Ca^{2+}]_i$ conditions are used. Similar changes in $[Na^+]_i$ were also observed for OAG-induced PKC activation (also see Belusa *et al.* 1997).

The mechanism by which Ca^{2+} modulates the regulation of NKA by PKA/PKC remains to be elucidated. This study shows that the state of phosphorylation of the α subunit by PKA/PKC is not influenced by variations in $[Ca^{2+}]_i$. It has also been shown that the inhibitory effect of PKC phosphorylation on purified NKA activity is neither influenced by Ca^{2+} nor by calmodulin. It is therefore likely that the modulatory effect of Ca^{2+} on phosphorylated NKA activity is effected via one or more Ca^{2+}-dependent intracellular signalling pathway(s).

The finding that $[Ca^{2+}]_i$ modulates the regulation of NKA activity by PKA and PKC has several important physiological implications. First, it implies that hormones activating PKA/PKC without concomitant increases in $[Ca^{2+}]_i$ may have an inhibitory effect on NKA activity, while hormones that both activate PKA/PKC and increase $[Ca^{2+}]_i$ may have a stimulatory effect. Second, in the active

Table 3. Effects of PKA or PKC activation on the activity of NKA in relation to Ca^{2+} conditions

NKA preparations	Experiment condition	Treatment	Effects on NKA	References
Microsomes or membranes from liver, kidney, heart, brain or pancreatic islets of rat, human and dog	Ca^{2+}-free buffers	cAMP or PKA	↓ ATP hydrolysis	Limas *et al.* 1973; Tria *et al.* 1974; Braughler & Corder, 1978; Lingham & Sen, 1982; Tung *et al.* 1990
Membranes from rabbit kidney or ciliary processes	Ca^{2+}-free buffer	cAMP or PKA	↓ ATP hydrolysis	Delamere *et al.* 1990
Rat renal tubule suspension	Ca^{2+}-free buffer	Forskolin	↓ATP hydrolysis	Hussian *et al.* 1997
Isolated rabbit iris–ciliary body	Ca^{2+}-free buffer	cAMP	↓ Rb^+ uptake	Delamere *et al.* 1990
Isolated rat renal tubules	Buffer 0·25 mM Ca^{2+}	cAMP	↓ ATP hydrolysis*	Fryckstedt & Aperia, 1992; Satoh *et al.* 1993; Holtbäck *et al.* 1998
Mouse fibroblast LTK^- cells	$[Ca^{2+}]_i$ not increased	cAMP	↓ ATP hydrolysis	Horiuchi *et al.* 1993
HeLa cells	$[Ca^{2+}]_i$ ~30 nM	cAMP	↓ Rb^+ uptake*	Middleton *et al.* 1990
Isolated guinea-pig myocytes	$[Ca^{2+}]_i < 150$ nM	PKA	↓ pump current	Gao *et al.* 1992
Isolated guinea-pig myocytes	$[Ca^{2+}]_i > 150$ nM	PKA	↑ pump current	Gao *et al.* 1992
Isolated rabbit renal tubules	Buffer 1·8 mM Ca^{2+}; low K^+ (0·1 mM) pretreatment	cAMP or forskolin	↑ transmembrane potential	Breton *et al.* 1994
Isolated shark rectal gland cells	Buffer 2·5 mM Ca^{2+}	cAMP	↑ Rb^+ uptake	Marver *et al.* 1986
Xenopus oocytes	Buffer Ca^{2+} 2 mM; K^+-free pretreatment and oocyte injection	cAMP	↑ pump current	Vasilets *et al.* 1992
Purified NKA from rat kidney and shark rectal gland	Ca^{2+}-free buffers	PKC	↓ ATP hydrolysis	Bertorello *et al.* 1991; Logvinenko *et al.* 1996
MDCK cells	Ca^{2+}-free buffer	PMA/OAG	↓ ATP hydrolysis	Shahedi *et al.* 1992
Isolated rat renal tubules	Buffer 0·25 mM Ca^{2+}	PDBu	↓ ATP hydrolysis	Bertorello & Aperia, 1989; Satoh *et al.* 1993
Isolated rat choroid plexus	Buffer 0·25 mM Ca^{2+}	PDBu	↓ ATP hydrolysis	Fisone *et al.* 1995
Cultured rat tubule cells	Buffer 0·4 mM Ca^{2+}	PMA	↓ Rb^+ uptake	Singh & Linas, 1997
A6 cells	Buffer 1·0 mM Ca^{2+}	PMA	↓ pump current	Beron *et al.* 1997
Isolated rat hepatocytes	$[Ca^{2+}]_i$ ~250 nM	PMA	↑Rb^+ uptake	Lynch *et al.* 1986
Cultured skeletal muscle	Buffer 0·7 mM Ca^{2+}; K^+-free pretreatment	Phorbol esters	↑ Rb^+ uptake	Sampson *et al.* 1994
Isolated rat renal tubules	Buffer 1·0 mM Ca^{2+}; osmolarity 400 mosmol kg^{-1}; K^+-free and low Rb^+ pretreatment	PDBu	↑ or no change in Rb^+ uptake/ATP hydrolysis	Feraille *et al.* 1995
Cultured human ciliary epithelium	Buffer 2·5 mM Ca^{2+}	PDBu	↑ Rb^+ uptake	Mito & Delamere, 1993
Cultured rabbit non-pigmented ciliary epithelium	Buffer 2·5 mM Ca^{2+}	PDBu	↑ Rb^+ uptake	Delamere *et al.* 1997

* The decreased NKA activity was abolished in the presence of agents that increase $[Ca^{2+}]_i$.
PDBu, 4-phorbol 12,13-dibutyrate.

state of some excitable tissues such as the beating heart, the exercising skeletal muscles and the firing neurons, the average $[Ca^{2+}]_i$ is expected to be quite high. Application of hormones or neurotransmitters to those tissues may be expected to lead to an increased activity of NKA, which has implications both for the intracellular ion homeostasis and the transmembrane potential. Third, the $[Ca^{2+}]_i$-dependent characteristic of regulation of NKA by

PKA/PKC may be important in the feedback control of NKA activity. Inhibition of NKA leads to an increase in $[Na^+]_i$ (Wong & Foskett, 1991; Blaustein, 1993). Increased $[Na^+]_i$ will attenuate the Na^+–Ca^{2+} exchanger (Blaustein, 1993), which results in increased $[Ca^{2+}]_i$. The resulting increase in $[Ca^{2+}]_i$ should counteract PKA/PKC-induced inhibition of NKA, allowing NKA activity to return toward resting levels, or even turning the direction to a stimulation or an oscillation (Wong & Foskett, 1991).

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