Diacylglycerol activation of protein kinase C results in a dual effect on Na$^{+}$,K$^{+}$-ATPase activity from intact renal proximal tubule cells

ALEJANDRO M. BERTORELLO
Department of Paediatrics, St Göran’s Children’s Hospital, Karolinska Institute, Box 12500, 11281 Stockholm, Sweden

Summary

This study evaluated the effect of L-l-oleoyl-2-acetoyl-sn-3-glycerol (OAG) on ouabain-sensitive Na,K-dependent oxygen consumption (Na,K-QO$_2$) in intact renal proximal tubule cells (RPTC). Basal Na,K-QO$_2$ (nmol O$_2$/mg protein per min) was 20.0±1.0. Incubation with 10 nM of OAG induced a dual effect on Na,K-QO$_2$, with an initial stimulation (maximal at 10 min, 37.1±5.0), followed by an inhibition (significant at 20 min, 16.3±1.0). No changes in ouabain-insensitive QO$_2$ were observed in any of the protocols. The effects were abolished by sphingosine, a protein kinase C inhibitor. Stimulation was abolished by amiloride 0.1 mM. Amiloride had no effect on Na,K-QO$_2$ at the concentration used. Stimulation was not potentiated by the sodium ionophore, amphotericine B, and the later inhibition was still observed in the presence of amphotericine B. The initial stimulation was attributed to an increase in sodium permeability, while the later inhibition was attributed to a direct effect on the Na,K-pump. Regulation of Na$^{+}$,K$^{+}$-ATPase activity by protein kinase C in intact RPTC can be accomplished by a direct effect on the protein or as a secondary effect consequent upon changes in intracellular sodium.

Key words: Na$^{+}$-gradient, Na$^{+}$-coupled transporters, Na$^{+}$/H$^{+}$ exchanger, protein kinase C isoforms, polar epithelial cells.

Introduction

The membrane-bound Na$^{+}$,K$^{+}$-ATPase couples the transport of sodium and potassium to the hydrolysis of ATP, thereby creating the driving force for sodium transport across the cell (Glynn, 1985; Skou, 1988). Short-term regulation of Na$^{+}$,K$^{+}$-ATPase activity by hormones (Bertorello and Aperia, 1990; Bertorello et al. 1990; Bertorello et al. 1988) and second messengers (Bertorello and Aperia, 1986a, b; Tung et al. 1990; Hughes et al. 1988; Beach et al. 1987) has been extensively studied. Moreover, activation of protein kinase C has been implicated (Bertorello and Aperia, 1986a, b) through a mechanism that appears to be mediated by protein phosphorylation (Bertorello et al. 1991).

Activation of protein kinase C in various tissues has been associated with a variety of cellular responses that are tissue specific (Nishizuka, 1986). In the renal tubular cells, activation of protein kinase C has been associated with an increase in the Na$^{+}$/H$^{+}$ exchanger activity (Mellas and Hammerman, 1986). In single permeabilized proximal tubule segments the activation of protein kinase C inhibits Na$^{+}$,K$^{+}$-ATPase activity (Bertorello and Aperia, 1989) and decreases net sodium transport (Baum and Hays, 1988). Stimulation of the Na$^{+}$/H$^{+}$ exchanger in renal proximal tubules and in non-renal tissues (Mellas and Hammerman, 1986) has been reported to occur within minutes, whereas inhibition of Na$^{+}$,K$^{+}$-ATPase activity and sodium transport occurred after a time-lag.

In order to determine whether activation of protein kinase C might have different effects on the apical and basolateral membranes and whether these effects may occur at different times, a preparation of cell suspension enriched in proximal tubules was used and the ouabain-sensitive Na,K-dependent oxygen consumption was determined in the presence of an endogenous activator of protein kinase C. This protocol makes it possible to study simultaneously both of the major determinants of transcellular sodium transport in the same cell preparation.

Materials and methods

Preparation of proximal tubule cell suspension

Male Sprague-Dawley rats (Ewos, Sodertalje, Sweden) weighing between 150 and 200 g were used in the experiments. After anaesthesia with Inactin (Byk-Golden Coblenz; 50 mg/kg, i.p.) the kidneys were removed and the cortex was isolated. Thereafter, the tissue was moistened on ice to a paste-like consistency. The cortical tissue was incubated with 0.075 mg/100 ml collagenase (Type I, Sigma) in 50 ml Dulbecco’s modified Eagle’s medium (DEMEM). Incubation was carried out at 37°C for 60 min. The solution was
continuously exposed to 95% O2/5% CO2. It was cooled on ice and poured through graded sieves (180-75-53-38 μm in pore size) to obtain a cell suspension. This suspension contains mostly proximal tubule cells (Seri et al. 1988). The cell suspension was washed three or four times by centrifuging at 100 g for 4 min in order to separate the remaining blood cells and traces of collagenase. No smooth muscle or endothelial cells were visible in the preparation by phase-contrast microscopy. The cells were used within two hours of preparation and kept on ice until studied. The quality of each preparation was monitored by microscopy and the viability was assessed by Trypan blue exclusion. The final pellet was resuspended in DEMEM to yield a protein concentration of approximately 3.5-5.0 mg/ml. Protein concentration was assessed by Trypan blue exclusion. The final pellet was resuspended in DEMEM to yield a protein concentration of approximately 3.5-5.0 mg/ml. Protein concentration was assessed by the Bradford's (Bradford, 1976) method, using a conventional dye reagent (Bio Rad Laboratories, Richmond, CA, USA).

Measurements of O2 consumption
Samples of cell suspension (500 μl) were transferred to a glass chamber containing 2.5 ml of DEMEM and maintained at 37°C by a circulating water jacket. Sodium butyrate (1 mM) was added to optimize mitochondrial oxidative metabolism to a maximal ADP-coupled and ADP-uncoupled rate (Harris et al. 1982). After 2 min equilibration, the drug or appropriate vehicle was added and the solution was mixed by means of slow stirring with a magnet (1 min). After different incubation times, the oxygen tension was recorded by an O2 electrode connected to a O2 meter (Yellow Spring Instruments, Yellow Springs, OH, USA) and was read by a digital computer. Ouabain (2×10^-3M) was used to determine the Na+,K+-ATPase-independent respiration. The addition of ouabain and other drugs, according to the different protocols, was done in a volume of <1% of the chamber to avoid potential dilution problems. The total O2 content of the incubation solution was never allowed to fall below 70%. When the measurements were completed the cell suspension was removed and the protein content was determined by Bradford's (Bradford, 1976) method.

Statistical analysis
Statistical comparison of the data was done by using the paired Student's t-test, or ANOVA when appropriate. P<0.05 values were considered significant.

Results
In this study we evaluated the effect of protein kinase C activation on the rate of Na,K-QO2 by intact renal proximal tubular cells (RPTC) in suspension. A large component of epithelial O2 must be consumed in order to supply the metabolic substrate for the Na+,K+-ATPase (which consumes ATP in order to transport sodium out of and potassium into the cell). To evaluate the effect of protein kinase C on Na,K-QO2, an endogenous activator of protein kinase C (L-1-oleoyl-2-acetoyl-sn, n-acetoyl-glycerol (OAG) was added to the cell suspension at a concentration of 10 nM, which is considered to be physiological (Fig. 1). Incubation was at 37°C and for different intervals. OAG was diluted in DMSO (final concentration <0.01%); therefore appropriate control was exercised. Basal Na,K-QO2 (nmol O2/mg protein per min) in RPTC was 20.0±1.0, n=6. Incubation of RPTC for as long as 30 min did not result in a decrease in Na,K-QO2 that would suggest cell damage. The presence of 10 nm OAG induced a biphasic effect on Na,K-QO2, an initial stimulation significant at 5 min (32±4.3, n=5, P<0.01) and maximal at 10 min (37.1±5.0, n=8, P<0.001), followed by an inhibition maximal at 20 min (16.3±1.0, n=8, P<0.025). The effect of OAG was, indeed, mediated by activation of protein kinase C. There was no non-ionic detergent-like effect, since the presence of 10^-6M sphingosine inhibited the stimulation (10 min) and inhibition (20 min) of Na,K-QO2 by 10^-7 M OAG (19.9±1.1, n=4 and 19.3±2.8, n=4, respectively). The inactive diacylglycerols neither inhibited nor stimulated Na,K-QO2 (not shown).

To determine whether OAG modulation of Na,K-QO2 was secondary to changes in mitochondrial oxidative phosphorylation, the effect of OAG was evaluated in the presence and the absence of FCCP (2×10^-5M) in order to uncouple respiration from ATP production. Under these circumstances, mitochondrial respiration is independent of cellular ADP and therefore not influenced by Na+, K+-ATPase activity. In the presence of FCCP QO2 was 71.0±5.3 (n=4) and it was not influenced by 10 min (71.8±5.7, n=4) or 20 min (68.0±3.0, n=4) incubation with 10 nM OAG. These results indicate that the effect of OAG was due to changes in Na+,K+-ATPase-dependent oxygen consumption.

The initial stimulation was dose-dependent, significant with 1 nM OAG (24.6±1.0, n=5, P<0.05), while the late (20 min) inhibition was significant with 10 nM OAG (16.3±1.0, n=8, P<0.025), a concentration that is comparable to the dose-effect obtained with phorbol 12,13-dibutyrate in single microdissected renal proximal tubule segments (Bertorello and Aperia, 1989a,b).

The intracellular sodium concentration is approximately 15 mM, a concentration substantially lower than that required for half-maximal activation of the Na+,K+-ATPase in vitro. Any change in intracellular sodium will therefore change the Na+,K+-ATPase...
activity. Considering these possibilities, the Na+,K+-ATPase can be regulated directly or indirectly via an effect on sodium permeability. To separate these two components, Na,K-QO2 was evaluated in the presence (Na+-loaded) and absence (normal intracellular Na+) of a polyene antibiotic, amphotericin B (10 μg/ml; Fig. 2). The early stimulation was due to an increase in sodium permeability. Incubation with 10 nM OAG as well as with amphotericin B-increased Na,K-QO2. However, these two drugs when incubated together did not result in a greater stimulation. In addition, the stimulatory effect of 10 nM OAG at 10 min was abolished by 10^-5 M amiloride, a concentration that itself did not affect Na+,K+-ATPase activity (data not shown). Moreover, the stimulatory effect of 10 nM OAG at 10 min was prevented by an increase in cellular cyclic AMP with 10^-5 M forskolin. Na,K-QO2 in the presence of 10 nM OAG was 139% of the control value and, together with 10 μM forskolin, it was 83% of the control value. Forskolin itself reduces Na,K-QO2 to 87% of the control.

Incubation of RPTC with OAG for 20 min resulted in a significant inhibition of Na,K-QO2. In order to determine whether OAG inhibited Na,K-QO2 by reducing Na+ influx via the Na+/H+ exchanger, the effect of 10 nM OAG in the presence of amphotericin B (Fig. 3) was evaluated in the next protocol. Amphotericin B significantly stimulated Na,K-QO2 (P<0.001). In this situation, where sodium is no longer rate-limiting, OAG inhibited Na,K-QO2, which indicates that inhibition of Na+,K+-ATPase activity at 20 min is accomplished by a direct effect on the enzyme rather than by changes in luminal sodium permeability.

Discussion

It has previously been demonstrated that inhibition of Na+,K+-ATPase activity by endogenous (OAG) and exogenous (PDBu) activators of protein kinase C occurred in permeabilized renal proximal tubules (Bertorello and Aperia, 1989a,b). These studies were done in single permeabilized proximal tubule segments, using a methodological approach that examined the Na+,K+-ATPase activity at Vmax for its major substrates: sodium, potassium and ATP. This procedure eliminates all the Na+-coupled transport mechanisms secondary to a Na+ gradient. In this study, by measuring ouabain-sensitive QO2 in freshly prepared proximal tubule cells it was possible to evaluate the role of the Na+-coupled transporters (apical permeability) in the regulation of Na+,K+-ATPase activity. The study confirms that indeed the Na+,K+-ATPase activity is directly down-regulated by protein kinase C. It also shows that the Na+,K+-ATPase activity can also be stimulated by protein kinase C. However, in this cell type the stimulation is not accomplished by a direct effect on the Na+,K+-ATPase molecule.

Diacylglycerol did not modify ouabain-insensitive QO2. The energy source for the Na+,K+-ATPase activity is provided by ATP. ADP-uncoupled respiration was also not modified by diacylglycerol, demonstrating that no sodium transport-independent mechanism was indirectly modulated.

This study demonstrates that activation of protein kinase C in RPTC segments generates two types of effects, which are different in nature and occur at different times.

The Na+,K+-ATPase activity is initially stimulated as a consequence of an increase in intracellular sodium due to high permeability throughout the Na+/H+ exchanger. This contention is supported by the following observations: (i) in the presence of amphotericin B, when sodium permeability was maximal, diacylglycerol did not elicit any further increase in Na+,K+-ATPase activity. (ii) Moreover, the effect was prevented by amiloride, an inhibitor of the Na+/H+ exchanger, which at the concentration used did not affect the Na+,K+-ATPase activity. (iii) Exogenously added cyclic AMP or a hormonal increase in cellular levels of cyclic AMP (Weinman et al. 1987) have been shown to inhibit the Na+/H+ exchanger. In the presence of forskolin (which stimulates adenylate

Activation of protein kinase C also inhibits the Na⁺,K⁺-ATPase activity, and the evidence suggests that the effect is accomplished by a direct effect of protein kinase C on the Na⁺,K⁺-pump. Amphotericin B, a polyelectrolyte antibiotic, increases sodium permeability, which increases the intracellular sodium concentration. In this situation where sodium is no longer rate-limiting, diacylglycerol inhibits Na⁺,K⁺-ATPase activity. Diacylglycerol did not inhibit Na⁺,K⁺-ATPase activity in basolateral membranes or when a pure preparation of Na⁺,K⁺-ATPase was used (Bertorello and Aperia, 1989a,b). The case for a direct effect of protein kinase C on the Na⁺,K⁺-ATPase molecule is further supported by recent experiments (Bertorello et al. 1991) in which the Na⁺,K⁺-ATPase α-subunit is phosphorylated by pure protein kinase C in vitro. Moreover, phosphorylation of the α-subunit was associated with a decrease in its catalytic activity.

Inhibition of Na⁺,K⁺-ATPase activity becomes apparent after 15 min of exposure to the protein kinase C activator. However, it seems that this effect occurs earlier. In the presence of high cellular cyclic AMP levels (incubation with forskolin), OAG inhibition (although not significant) of Na⁺,K⁺-ATPase activity already occurred at 5 min. These observations suggest that when the activity of the Na⁺/H⁺ exchanger was suppressed by cyclic AMP, activation of protein kinase C elicits its inhibitory effect on Na⁺,K⁺-ATPase activity. This phenomenon may suggest that diacylglycerol will more potently activate the mechanism that triggers the activation of the Na⁺/H⁺ exchanger. This observation by no means represents a situation that occurs physiologically. However, isolated cells exposed to a situation where all the protein kinase C is activated, will respond in a manner calculated to maintain cell homeostasis.

It may also be the case that diacylglycerols exert a different time- and dose-dependent translocation (as depicted in Fig. 4) of protein kinase C to either the brush border or the basolateral membranes, which is responsible for the dual effect on Na⁺,K⁺-ATPase activity in renal epithelial cells. Supporting this idea are recent findings, where carbachol induced a specific translocation of protein kinase C to the brush border membrane of rabbit ileal cells (Cohen et al. 1991) and also studies done in platelets, where different protein kinase C isoforms were translocated to the membrane depending on the concentration of phorbol esters (Crabos et al. 1991). Another possibility to be considered, is that the protein kinase C translocation is deactivated faster in the brush border membrane than in the basolateral membrane. The efficiency of a physiological agonist will, however, depend not on its potency but probably on its selectivity to translocate the kinase to either membrane.

Finally, protein kinase C exists in different isoforms, which are unevenly distributed within tissues (Nishizuka, 1988) and probably within the same cell (Otte et al. 1991). To date, no evidence of the existence of different isoforms within the kidney has been reported. The finding that the response occurred at different concentrations of OAG (stimulation at 1 nM and inhibition at 10 nM) indicates that different isoforms are probably responsible for the regulation of different transport systems and that these isoforms may have various degrees of sensitivity to regulators.

References


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