Vol. 200, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1994 Pages 569-576

ACUTE ELEVATION OF INTRACELLULAR pH TRANSLOCATES PROTEIN KINASE C ACTIVITIES IN RAT PINEALOCYTES

Anthony K. Ho* and Constance L. Chik

Department of Physiology and Department of Medicine
University of Alberta
Edmonton, Alberta T6G 2H7, Canada

Received February 2, 1994

SUMMARY: The relationship between elevation of intracellular pH and activation of protein kinase C in rat pinealocytes was investigated in rat pinealocytes. It was found that direct elevation of intracellular pH alone by ammonium chloride or methylamine translocated protein kinase C activities in a dose-dependent manner. This translocation was blocked by EGTA, suggesting a requirement of Ca^{2+} for the process. However, elevation of intracellular pH by methylamine had no significant effect on intracellular Ca^{2+} or diacylglycerol content, suggesting the translocation was not due to increased intracellular Ca^{2+} or diacylglycerol contents. These data suggest that elevation of intracellular pH alone can trigger activation of protein kinase C through a mechanism distinct from increases in diacylglycerol or intracellular Ca^{2+}.

In rat pinealocytes, the accumulation of cyclic nucleotides is regulated by a dual receptor system involving the synergistic interaction of both α_1- and β-adrenoceptor regulated processes (1,2). The β-adrenergic activation appears to be an absolute requirement and α_1-adrenergic stimulation acts to potentiate this response. At the post-receptor level, protein kinase C (PKC)[1] plays a central role in this potentiation mechanism (3,4,5). Recently it was found that activation of α_1-adrenoceptors, apart from activating PKC, also leads to cytoplasmic alkalinization through activation of the Na^+/H^+ antiport system (6). Furthermore, it was also demonstrated that changes in intracellular pH (pHi) or inhibition of the Na^+/H^+ antiport can have a significant modulating effect on the

*To whom correspondence should be addressed at Room 7-26 MSB, Department of Physiology, University of Alberta, Edmonton, Alberta T6G 2H7, Canada.
Fax: (403) 492-8915.

The abbreviations used are: protein kinase C, PKC; intracellular pH, pHi; 2'7'-Bis(carboxy-ethyl)-5(6)-carboxyfluorescein, BCECF; diacylglycerol, DG; intracellular Ca^{2+}, $[Ca^{2+}]i$.

0006-291X/94 $5.00

adrenergic stimulated cAMP and cGMP accumulation (6,7). In view of the importance of the PKC in the regulation of cyclic nucleotides in the rat pinealocyte, these earlier results suggest that changes in pHi may affect PKC activation. In the present study, the role of pHi in the activation of PKC in rat pinealocytes was investigated by elevating pHi directly using ammonium chloride and methylamine (8).

METHODS

Norepinephrine, digitonin, diacylglycerol, phospholipids, histone (type III), ATP, EGTA, ammonium chloride and methylamine were obtained from Sigma (St. Louis, MO). [γ-^{32}P]ATP was from ICN (Irvine, CA). The acetoxymethyl esters of 2'7'-Bis(carboxy-ethyl)-5(6)-carboxyfluorescein (BCECF) and fura-2 were purchased from Molecular Probes Inc. (Eugene, OA). Culture medium was purchased from Gibco (Grand Island, NY). Other chemicals were obtained from commercial sources.

Pinealocytes were prepared from male Sprague-Dawley rats (200gm) by trypsinization as previously described (6,7,8). Cells were suspended in Dulbecco's modified Eagle medium containing 10% fetal calf serum and maintained (37°C) for 18 hr under 95% air and 5% carbon dioxide before experiment. For the [^{3}H]diacylglycerol (DG) measurement, [^{3}H]arachidonic acid (240Ci/mmol; 10μCi/ml) was present during this incubation period.

Intracellular pH and intracellular Ca^{2+} ([Ca^{2+}]i) were determined, in a SLM Aminco DMX1000 fluorescence spectrophotometer, using cells loaded with the fluorescent pH and Ca^{2+} indicator, BCECF and fura-2 respectively as described (6,8). The pHi of pinealocytes was measured at 37°C by monitoring the ratio of the fluorescence emission signal at 525nm, with the excitation wavelengths set at 500nm and 450nm. For [Ca^{2+}]i, the excitation wavelengths used were 380nm and 340nm and the emission signal was monitored at 510nm. Calibration of the pHi and free Ca^{2+} concentration were performed as previously described (6,8).

In the study of PKC translocation, the pinealocytes were collected after drug treatment by centrifugation and placed in a 0°C ice-water bath. For the determination of the PKC distribution, pinealocytes were first permeabilized by incubation (7 min, 4°C) in 50 μM digitonin in buffer A (20mM TRIS-HCl, containing 2mM EDTA, 0.5mM EGTA, 2mM phenylmethylsulphonyl fluoride, 0.5mg/ml leupeptin, pH 7.5) (16,17). After permeabilization, the cytoplasmic and the membrane fractions were separated by centrifugation. The membrane fraction was then solubilized in 0.05% NP-40 in buffer A. PKC activity was measured in duplicate as described previously using histone type III as substrate (3,7) and was calculated from the difference in ^{32}P incorporated into histone in the presence and absence of added phospholipids.

To study [^{3}H]DG generation, [^{3}H]arachidonic acid labelled cells were washed twice and treated with drugs. R59022, a DG kinase inhibitor, when present, was added 2 min before addition of other drugs. Drug treatment was terminated by addition of ice cold chloroform/methanol. [^{3}H]DG contents were isolated by TLC procedure and measured as previously described (9).

Data are presented as the mean ± SEM from three aliquots of cells and was analyzed by Student's unpaired t-test. Paired t-test was used for the analysis of pHi and [Ca^{2+}]i measurements.

RESULTS

Effects of ammonium chloride and methylamine on pHi: Using BCECF loaded cells, the effects of ammonium chloride and methylamine on pHi were determined. At extracellular

pH of 7.40, the resting pHi of pinealocytes was 7.16 ± 0.03. Addition of ammonium chloride produced a rapid alkalinizing effect on cytosolic pH which peaked at 20 sec (Fig. 1), reversed partially and stabilized at a higher pHi 2 min post treatment. This alkalinizing effect of ammonium chloride was dose-related (Table 1). Addition of methylamine also produced a sustained and dose-dependent elevation of pHi but without the initial rapid elevation (Fig. 1 and Table 1). Based on these results, the concentration of ammonium chloride and methylamine chosen for the following studies were 40mM and 2mM respectively. At these concentrations, the increases in pHi induced by these agents were similar to that stimulated by NE (Fig. 1), the physiological regulator of pineal function.

Effects of ammonium chloride and methylamine on the subcellular distribution of PKC activity: Analysis of the subcellular distribution of PKC in digitonin-treated control cells indicated that less than 10% of total PKC activity was present in the membrane pellet after permeabilization (Table 1). Treatment with ammonium chloride (40mM) or methylamine (2mM) for 5 min increased membrane-associated PKC activity by 80% and 100% respectively ($P < 0.05$; Table 1). Both effects were dose-related.

Role of $[Ca^{2+}]i$ on the ammonium chloride and methylamine mediated PKC translocation: To examine the issue whether influx of Ca^{2+} is important for the PKC translocating effect of ammonium chloride and methylamine, two approaches were used. First, their effects on $[Ca^{2+}]i$ were determined in fura-2 loaded cells. Addition of ammonium chloride elevated $[Ca^{2+}]i$ from 151 ± 12nM to 178 ± 10nM (Fig. 2). This effect of ammonium chloride was blocked by 10μM nifedipine suggesting voltage-dependent Ca^{2+} channel may be involved. In contrast, methylamine (2mM) had no effect on $[Ca^{2+}]i$ (Fig. 2).

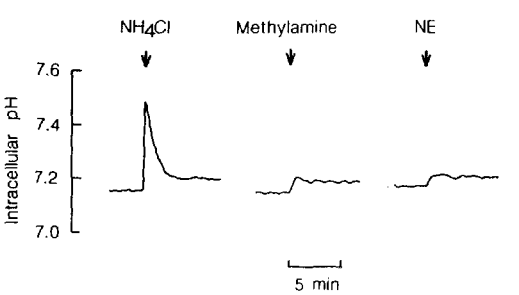

Figure 1. Effect of ammonium chloride, methylamine and norepinephrine on pHi of rat pinealocytes. Rat pinealocytes were prepared, loaded with the fluorescent pH indicator BCECF and washed three times before measurement. Fluorescence signal was calibrated with nigericin/KCl. After defining the base line pHi, ammonium chloride (NH_4Cl; 40mM), methylamine (2mM) and norepinephrine (NE; 10^{-5}M) were added as indicated by the arrows. The traces are representatives of at least three separate experiments.

Table 1. Effect of norepinephrine, ammonium chloride and methylamine on the pHi and on distribution of PKC activity in the rat pinealocyte. For pHi determination, pinealocytes were loaded with the fluorescence pH indicator BCECF. For measurement and calibration of pHi see "Methods". For PKC measurement, pinealocytes were treated for 6 min with drugs, permeabilized and followed by centrifugation which separated the cytoplasmic from the membrane fractions. PKC activity was measured in membrane and cytosolic fractions after solubilization. Each value represents the mean ± SEM of determinations done in duplicate on three samples of cells.

Treatment	pHi[1]	Protein kinase C activities	
		cytosolic	membrane
		(pmol/6min/10^6 cells)	
Control	7.09 ± 0.007	1897 ± 37	233 ± 28
Norepinephrine (10μM)	7.16 ± 0.018*	1733 ± 83	370 ± 36*
Ammonium chloride:			
20mM	7.13 ± 0.009*	1744 ± 83	389 ± 36*
40mM	7.19 ± 0.015*	1653 ± 55*	423 ± 68*
Methylamine :			
1mM	7.10 ± 0.004	1733 ± 88	332 ± 56*
2mM	7.20 ± 0.013*	1573 ± 109*	446 ± 77*

[1] Values represent the steady levels, 3 min after drug addition.
* Significantly different from control ($P < 0.05$).

To determine whether inhibition of Ca^{2+} influx has any effect on the translocation of PKC stimulated by pHi elevating agents, it was found that addition of 10μM nifedipine, which completely blocked the ammonium chloride-mediated elevation of $[Ca^{2+}]i$ (Fig.2), only reduced the ammonium chloride-stimulated translocation of PKC by less than 30%

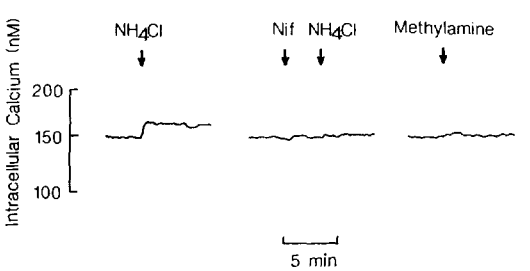

Figure 2. Effects of ammonium chloride and methylamine on $[Ca^{2+}]i$. Rat pinealocytes were prepared and loaded with the fluorescent indicator fura-2. Ratio of the fluorescence emission signal at 510nm, excited at 380 and 340nm, was continuously recorded and calibrated as described. After defining the base line pHi, ammonium chloride (NH_4Cl; 40mM), nifedipine (Nif; 10^{-5}M) and methylamine (2mM) were added as indicated by the arrows. The traces are representative of at least three separate experiments.

Table 2. Effect of nifedipine and EGTA on ammonium chloride and methylamine stimulated distribution of PKC activity in the rat pinealocyte. Pinealocytes were aliquoted (4 x 105 cells/0.5ml) and treated for 5 min with drugs which had been dissolved in water or DMSO. Cells were then collected by centrifugation and permeabilized. This was followed by centrifugation which separated the cytoplasmic from the membrane fractions. PKC activity was measured in membrane and cytosolic fractions after solubilization. Only membrane associated activities are present. Each value represents the mean ± SEM of determinations done in duplicate on three samples of cells.

Treatment	Protein kinase C activity
	(pmol/6min/10^6cells)
Control	249 ± 13
Nifedipine (10μM)	214 ± 18
EGTA (5mM)	186 ± 13
Ammonium chloride (40mM)	458 ± 36*
+ Nifedipine (10μM)	376 ± 26*
+ EGTA(5mM)	224 ± 27**
Methylamine (2mM)	353 ± 27*
+ Nifedipine (10μM)	381 ± 26*
+ EGTA(5mM)	230 ± 19**

* Significantly different from the control group ($P < 0.05$).
** Significantly different from the ammonium chloride or methylamine treated group ($P < 0.05$).

(Table 2). Nifedipine, however, had no effect on the PKC translocation stimulated by methylamine. Addition of EGTA, which inhibited the translocation stimulated by ionomycin (9), also inhibited the translocation stimulated by ammonium chloride or methylamine.

Effects of ammonium chloride and methylamine on [^{3}H]DG production: The possibility that the translocation of PKC stimulated by elevation of pHi may involve an enhanced DG accumulation was assessed by determining whether ammonium chloride and methylamine caused any significant increase in [^{3}H]DG production. Since DG generation in the pinealocytes is known to be rapidly metabolized by DG kinase (10), in the present study, experiments were done in the presence and absence of an inhibitor of DG kinase, R59022 (10,11). In the presence of the DG kinase inhibitor, there was a significant increase in [^{3}H]DG accumulation in control cells (a 6-fold increase after 30 min) and NE caused a further 2-fold increase in [^{3}H]DG accumulation. However, neither ammonium chloride nor methylamine had any significant effect on [^{3}H]DG accumulation under the same condition (Table 3).

Table 3. Effect of ammonium chloride and methylamine on diacylglycerol production in rat pinealocytes. Pinealocytes were prelabelled with [^{3}H]arachidonic acid (240Ci/mmol; 10μCi/ml) for 18 hr. Cells were aliquoted (1 x 10^6 cells/0.25ml) and treated for 5 min with drugs which had been dissolved in water. The incubation which was for 30 min at 37°C was terminated by addition of 0.95ml chloroform/ methanol (1:2; v/v), the lipids were extracted, and [^{3}H]diacylglycerol was isolated by TLC. Each value represents the mean ± SEM of determinations done on at least four samples of cells.

Treatment	Diacylglycerol content	
	Without R59022	With R59022
	(dpm/10^6cells)	
Control	1589 ± 172	9872 ± 429
Ammonium chloride (40mM)	1606 ± 120	9761 ± 748
Methylamine (2mM)	1649 ± 187	10143 ± 891
Norepinephrine (10 μM)	1685 ± 194	16424 ± 830*

* Significantly different from the control group ($P < 0.05$).

DISCUSSION

The purpose of these studies was to determine whether pHi had any effect on the translocation of PKC. This issue is of interest because it is known that activation of α_1-adrenoceptors produces a sustained elevation of pHi by activating the Na^+/H^+ antiport (6) and that these changes in pHi have a significant influence on the adrenergic-stimulated cAMP and cGMP accumulation (7,9). The results of the present experiments provide a clear indication that redistribution of PKC can be triggered by an elevation of pHi.

The mechanism through which pHi stimulates translocation of PKC appears to be different from other established mechanisms such as increases in $[Ca^{2+}]i$ (9) or DG production (12,13). Although ammonium chloride caused a small increase in $[Ca^{2+}]i$, methylamine, which had no effect on the $[Ca^{2+}]i$, was equally effective in stimulating PKC activation. Furthermore, while nifedipine completely blocked the ammonium chloride-mediated elevation of $[Ca^{2+}]i$, this Ca^{2+} channel blocker only had a limited effect on the ammonium chloride-mediated PKC activation. Thus it seems reasonable to suspect that the translocation of PKC stimulated by elevating pHi is independent of changes in $[Ca^{2+}]i$. However, it is also clear that Ca^{2+} is required for the process since treatment with EGTA abolished the effect of methylamine or ammonium chloride. It is also unlikely that elevation of pHi and generation of DG share a common pathway in activating PKC. This is based on the finding that neither methylamine nor ammonium chloride led to a significant generation of DG even in the presence of a DG kinase inhibitor. The absence of any significant effects of these agents on [^{3}H]DG production contrasts with the rapid effects these agents have on PKC translocation.

One possible mechanism through which elevated pHi translocates PKC may involve activation of phospholipase A_2 and the subsequent generation of arachidonic acid. In lymphocytes, it has been shown that phospholipase A_2 can be stimulated by elevation of pHi (14). Whether elevation of pHi will also activate phospholipase A_2 in rat pinealocytes remains to be determined. However, it has been shown that α_1-adrenergic activation, which elevates pHi (6), also stimulates phospholipase A_2 in rat pinealocytes resulting in the generation of arachidonic acid (15). The observation that Ca^{2+} is required for the increased pHi-mediated activation of PKC is also consistent with this interpretation since the α_1-adrenergic activation of PLA_2 is a Ca^{2+}-dependent process (15). Such a mechanism of generating arachidonic acid may cause activation of the γ-subspecies of PKC (16,17).

A relationship between increased pHi and activation of PKC may have a significant implication in the regulation of cyclic nucleotides in rat pinealocytes. The involvement of PKC in the regulation of both cAMP and cGMP responses has been established (3,4). Therefore, an effect of increasing pHi on activation of PKC may explain some of our earlier observations. For instance, the potentiating effect of ammonium chloride on adrenergic-stimulated cAMP and cGMP responses (8). In view of the importance of cAMP in the synthesis of the pineal hormone melatonin, these results provide additional evidence that pHi is an important regulator of the physiological function in the rat pineal gland.

As a final point, we would like to focus on the general implications of the evidence that an increase in pHi alone can trigger redistribution of PKC in the intact cell. This is of importance since it emphasizes that PKC may be activated in many systems by transmitters, hormones or other agents which elevate pHi but may have no other direct effect. Thus, pHi might be the critical element regulating the degree of membrane association of PKC.

ACKNOWLEDGMENTS

This work was supported by a grant from the Medical Research Council of Canada to A.K. Ho. C.L. Chik is a clinical investigator of the Alberta Heritage Foundation for Medical Research.

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