ELSEVIER

European Journal of Pharmacology 294 (1995) 11-15

ejp

Role of the Na^+-Ca^{2+} and Na^+-H^+ antiporters in prolactin release from anterior pituitary cells in primary culture

Gianfranco Di Renzo [a,c,*], Salvatore Amoroso [a], Antonella Bassi [a], Alessandro Fatatis [a], Mauro Cataldi [a], Anna Maria Colao [b], Gaetano Lombardi [b], Lucio Annunziato [a]

[a] *Section of Pharmacology, Department of Neuroscience, School of Medicine, 'Federico II' University of Naples, Via S. Pansini 5, 80131 Naples, Italy*
[b] *Department of Endocrinology, School of Medicine, 'Federico II' University of Naples, Via S. Pansini 5, 80131 Naples, Italy*
[c] *School of Pharmacy, University of Reggio Calabria, Catanzaro, Italy*

Received 2 March 1995; revised 8 August 1995; accepted 11 August 1995

Abstract

2',4'-dimethylbenzamilamiloride (DMB), a somewhat selective inhibitor of the Na^+-Ca^{2+} exchanger, in concentrations of 10, 30 and 100 μM did not produce any significant effect on baseline prolactin release from anterior pituitary cells in primary culture. When prolactin secretion was stimulated by the inhibitor of the Na^+-K^+-ATPase, ouabain, that activates the Na^+-Ca^{2+} exchanger as a Ca^{2+}-influx pathway, DMB was able to produce inhibition of prolactin secretion. 5-(*N*,*N*-hexamethylene) amiloride (HMA), another amiloride analog which specifically inhibits the Na^+-H^+ antiporter and has no inhibitory activity on the Na^+-Ca^{2+} exchanger, at the concentrations of 0.1, 1 and 10 μM, did not affect basal prolactin release whereas it significantly reduced prolactin release stimulated by thyrotropin releasing hormone (TRH) (1 μM). These results suggest that the Na^+-Ca^{2+} antiporter is involved in the process of prolactin release elicited by the inhibition of the Na^+-K^+-ATPase whereas the Na^+-H^+ antiporter is involved in the prolactin secretion elicited by TRH.

Keywords: Prolactin; Na^+-Ca^{2+} exchanger; Na^+-H^+ antiporter; Amiloride analog

1. Introduction

It has been extensively demonstrated that changes of Ca^{2+} and H^+ intracellular concentrations in different cell types may affect many physiological processes (Carafoli, 1987; Frelin et al., 1988). Cytosolic Ca^{2+} ions, in particular, play a crucial role in the regulation of secretory processes in most cells, including those of the anterior pituitary (Childs et al., 1987; Kolesnick et al., 1984; Kraicer and Spence, 1981; Tan and Tashjian Jr., 1984). It is now generally accepted that, among the plasma membrane systems that regulate Ca^{2+} intracellular concentrations, the Na^+-Ca^{2+} antiporter can mediate Ca^{2+} fluxes across the plasma membrane in a bidirectional way, coupling Ca^{2+} extrusion to Na^+ influx and vice versa (Carafoli, 1987). Although the activity of the Na^+-Ca^{2+} antiporter has been characterized both in plasma membrane vesicles prepared from pituitary GH_3 cells (Kaczorowski et al., 1985) and, more recently, in whole GH_4C_1 cells (Tornquist and Tashjian Jr., 1989), the relationship between the changes in its activity and the release of prolactin from anterior pituitary cells has not yet been examined. In addition, it has been shown that changes in intracellular pH (pH_i), due to modification of the activity of the Na^+-H^+ antiporter, may occur upon stimulation of thyrotropin releasing hormone (TRH) receptors (Hallam and Tashjian Jr., 1987), the activation of which is known to elicit prolactin release. Furthermore, evidence has been provided that pH_i modifications may affect the permeability of Ca^{2+} channels (Nachsen and Drapeau, 1988) and Ca^{2+} metabolism (Siffert and Akkerman, 1987).

The recent development of amiloride analogs which bear: (1) substituents on the guanidino nitrogen atom and therefore behave as specific inhibitors of the Na^+-

* Corresponding author. Section of Pharmacology, Department of Neuroscience, School of Medicine, 'Federico II' University of Naples, Via S. Pansini 5, 80131 Naples, Italy. Tel.: 0039-81-7463323; fax: 0039-81-7463323.

SSDI 0014-2999(95)00505 6

Ca^{2+} antiporter (Kaczorowski et al., 1985) or (2) substituents on the 5-amino nitrogen atom of the pirazine ring, therefore proved to be of great effectiveness to inhibit the Na^+-H^+ exchange system (Vigne et al., 1984), allowed the study of the possible involvement of these two plasma membrane antiporters in the secretion of prolactin from anterior pituitary cells in primary culture.

2. Materials and methods

Adult female Wistar rats (200–250 g) were kept under controlled conditions of light (14 h of light, 10 h of darkness; light on 0:6.00–20:00) and temperature (22°C). Food and water were supplied ad libitum.

The animals were killed by decapitation, the pituitary glands were removed and the posterior lobe was discarded. Anterior pituitary cells were dispersed enzymatically, according to the method of Vale et al. (1972) and plated in RPMI 1640 medium containing 7.5% horse serum, 2.5% fetal calf serum, 50 μg/ml penicillin, 50 μg/ml streptomycin and 2.5 μg/ml fungizone, at a density of 3×10^5 cells/ml in multiple well Flow dishes.

After 4 days in culture in a humified atmosphere of 95% air-5% CO_2, the cells were washed and incubated for 30 min in a medium containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose and 5 mM Hepes, and adjusted to pH 7.4 with 1 M Tris. Drugs to be tested were added to the incubation medium in a small volume and the same volume of diluent was added to control dishes. At the end of the incubation period, the medium was aspirated and stored at −20°C until assayed for prolactin concentrations. Cell viability was found to be 95–98% when verified with trypan blu at the end of the incubation period.

The concentration of prolactin in the medium was assayed with a double antibody radioimmunoassay (Niswender et al., 1969) using reagents supplied by NHPP, NIDDK, NICHHD, USDA. All samples were assayed within the same radioimmunoassay to avoid possible inter-assay variation. The intra-assay coefficient of variation was 2%.

The data were statistically evaluated by one-way analysis of variance (ANOVA) and the Newman-Keuls test.

The following drugs were used: 2',4'-dimethylbenzamilamiloride (DMB) and 5-(*N*,*N*-hexamethylene) amiloride (HMA), synthesized and supplied by Dr. Cragoe (Nacogdoches, TX). These drugs were prepared as stock solution in dimethylsulfoxide and were diluted direcly in the incubation medium and the maximal dimethylsulfoxide concentrations (1%) did not alter prolactin concentrations in this study. Ouabain was purchased from Sigma Chemical Italia (Milano). RPMI 1640 medium was purchased from Flow Laboratories (Irvin, Scotland).

3. Results

3.1. Effect of the inhibition of the Na^+-Ca^{2+} exchanger on basal and ouabain or TRH-induced prolactin release from anterior pituitary cells

DMB, a somewhat selective inhibitor of Na^+-Ca^{2+} exchanger, devoid of any inhibiting effect on the Na^+-H^+ antiporter (Kaczorowski et al., 1985; Taglialatela et al., 1990), in concentrations of 10, 30 and 100 μM did not produce any significant effect on baseline prolactin release (data not shown). By contrast, when prolactin secretion was stimulated by the inhibitor of the Na^+-K^+-ATPase, ouabain, which activates the Na^+-Ca^{2+} exchanger as a Ca^{2+} influx pathway (Blaustein, 1985), the Na^+-Ca^{2+} exchanger inhibitor, DMB (30 and 100 μM), was able to produce inhibition of anterior pituitary hormone secretion (Fig. 1).

Since it has been reported that polyphosphoinositide breakdown with IP_3 formation can activate the Na^+-Ca^{2+} exchanger in other cellular systems (Fraser and Sarnacki, 1992) the effect of the Na^+-Ca^{2+} exchange inhibitor was tested on the stimulation of prolactin release evoked by the IP_3-linked endogenous agonist TRH. DMB, even at the highest concentration

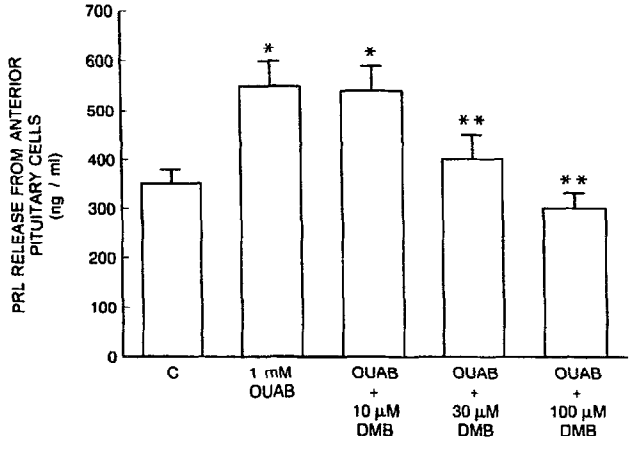

Fig. 1. Effect of different concentrations of DMB on ouabain-induced prolactin secretion from anterior pituitary cells. Cells of the control group were exposed to an incubation medium in which the concentration of K^+ ions was 5 mM. The incubation period was 30 min. Ouabain and the different concentrations of DMB were added at the same time. Each bar represents the mean ± S.E. of six values, obtained in two independent experiments. * $P < 0.05$ versus control group. ** $P < 0.05$ versus ouabain group.

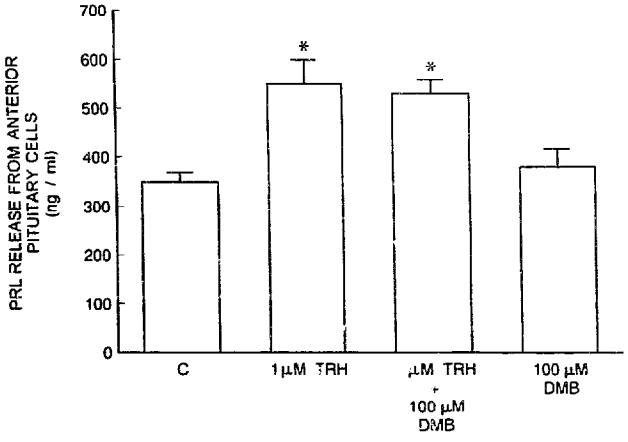

Fig. 2. Effect of DMB on prolactin release stimulated by TRH. Cells of the control group were exposed to an incubation medium in which the concentration of K^+ ions was 5 mM. The incubation period was 30 min. DMB and TRH were added at the same time. Each bar represents the mean ± S.E. of six values obtained in two independent experiments. * $P < 0.05$ versus control group.

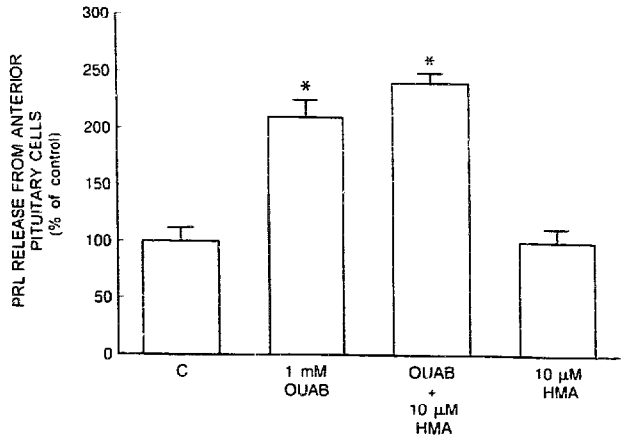

Fig. 4. Effect of HMA on prolactin release elicited by ouabain. Cells of the control group were exposed to an incubation medium in which the concentration of K^+ ions was 5 mM. The incubation period was 30 min. Ouabain and HMA were added at the same time. The values are expressed as percents of control values obtained in the absence of ouabain and HMA. Each bar represents the mean ± S.E. of six values that are from two independent experiments. * $P < 0.05$ versus control group.

of 100 μM, was unable to interfere with the releasing action of TRH (Fig. 2).

3.2. Effect of the inhibition of the Na^+-H^+ antiporter on basal and ouabain- or TRH-induced prolactin release

HMA, a specific inhibitor of the Na^+-H^+ antiporter (Simchowitz and Cragoe Jr., 1986), at the concentrations of 0.1, 1 and 10 μM, did not affect basal prolactin release (data not shown). Since it has been reported that TRH can induce changes in pH_i through activation of the Na^+-H^+ antiporter (Hallam and Tashjian Jr., 1987), the effect of the inhibitor of this antiporter on TRH-induced prolactin release was tested. HMA, at the concentrations of 1 and 10 μM, significantly reduced the prolactin release stimulated by 10 μM TRH (Fig. 3).

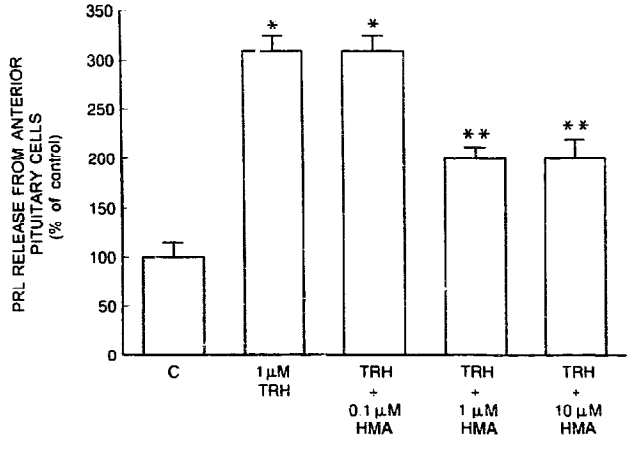

Fig. 3. Effect of different concentrations of HMA on prolactin release stimulated by TRH. Cells of the control group were exposed to an incubation medium in which the concentration of K^+ ions was 5 mM. The incubation period was 30 min. TRH and HMA were added at the same time. The values are expressed as percents of control values obtained in the absence of TRH and HMA and are from two independent experiments. Each bar represents the mean ± S.E. of six values. * $P < 0.05$ versus control group. ** $P < 0.05$ versus TRH group.

In order to provide further evidence for the specificity of the Na^+-H^+ antiporter inhibitor, which belongs to the chemical class of amiloride analogs and to exclude a hypothetical effect of this compound on the Na^+-Ca^{2+} antiporter, HMA was tested in conditions of activation of this exchange system elicited by ouabain. HMA, added at the highest concentration capable of inhibiting TRH-elicited prolactin release (10 μM), failed to induce any change of ouabain-stimulated prolactin release (Fig. 4).

4. Discussion

The results of the present study showed that two amiloride analogs belonging to two different subgroups of pirazine derivatives, DMB, a somewhat specific inhibitor of the Na^+-Ca^{2+} antiporter (Kaczorowski et al., 1985; Taglialatela et al., 1990) and HMA, that specifically blocks the activity of the Na^+-H^+ antiporter (Simchowitz and Cragoe Jr., 1986) did not induce any significant change of baseline prolactin release. These results suggest that Na^+-Ca^{2+} and Na^+-H^+ antiporters are not significantly involved in prolactin release in vitro under resting conditions. By contrast, DMB reduced ouabain-induced prolactin release. A possible explanation of these effects may be that, when the

transmembrane Na^+ electrochemical gradient is reduced, as occurs upon the inhibition of the Na^+-K^+ ATPase pump by ouabain (Repke and Shonfeld, 1984), the Na^+-Ca^{2+} antiporter works in a reverse way, leading to Ca^{2+} entry into the cells that is responsible for the stimulation of prolactin release. Therefore, the inhibition of the Na^+-Ca^{2+} antiporter by DMB may lead to the decrease of ouabain-induced PRL release. It should be emphasized that the concentrations of DMB (30 and 100 μM) effective to inhibit ouabain-induced prolactin release have been shown also to inhibit the Na^+-Ca^{2+} exchanger in pituitary vesicles (Kaczorowski et al., 1985). Further support for this interpretation is provided by the findings showing that preincubation with ouabain in a medium containing Na^+ causes an increase of $^{45}Ca^{2+}$ uptake in GH_3 cells (Kaczorowski et al., 1984) and stimulates the activity of the Na^+-Ca^{2+} antiporter in GH_4C_1 cells (Tornquist and Tashjian Jr., 1989). Since it has been shown that some amiloride analogs can also block L and T Ca^{2+} channel subtypes (Garcia et al., 1990), the possibility existed that DMB could inhibit ouabain-induced PRL release by preventing Ca^{2+} entrance through voltage-operated Ca^{2+} channels. However, this hypothesis does not appear likely, at least for the concentration of 30 μM. In fact, it has been shown that low concentrations (1–50 μM) of DMB, at which the inhibition of the Na^+-Ca^{2+} exchange is evident, have no significant effect on the inward Ca^{2+} current (Kaczorowski et al., 1985).

Since it has been demonstrated that TRH is able to cause, in pituitary clonal cell lines, modifications of pH_i as a consequence of the activation of the Na^+-H^+ antiporter (Hallam and Tashjian Jr., 1987), the possibility existed that changes in pH_i might interfere with the processes involved in TRH-elicited prolactin release.

The experiments performed in the present study with the Na^+-H^+ inhibitor, HMA, showed that the functional impairment of this antiporter can interfere with the processes which are involved in prolactin secretion elicited by TRH exposure. Concentrations of HMA effective to block TRH-induced prolactin secretion are in the range of those reported for the inhibition of Na^+-H^+ exchange in other cellular systems (Simchowitz and Cragoe Jr., 1986). Therefore, it is reasonable to hypothesize that the decrease of TRH-induced prolactin release is due to inhibition of the antiporter. The possibility that HMA can inhibit prolactin secretion evoked by TRH by interfering with other plasma membrane systems can be excluded since the inhibition of the Na^+-Ca^{2+} exchanger occurs at an order of magnitude about 2000-fold higher. In fact, the K_i of this compound for the Na^+-H^+ antiporter is 0.16 μM (Simchowitz and Cragoe Jr., 1986), whereas for the Na^+-Ca^{2+} antiporter it is 100 μM (Kaczorowski et al., 1985). On the other hand, the possible involvement of the Na^+-H^+ antiporter in TRH-induced prolactin secretion has been suggested by Mariot et al. (1993), who showed that amiloride is able to decrease TRH-stimulated prolactin release in GH_3B_6, a pituitary clonal cell line. The results of the present study, obtained with an amiloride analog more specific than the parent compound to inhibit the activity of the Na^+-H^+ antiporter, suggest that this exchanger is involved in TRH-induced prolactin release from prolactin-secreting cells in primary culture also.

The mechanism by which the blockade of the Na^+-H^+ exchanger inhibits TRH-stimulated prolactin secretion is not yet clear. Mariot et al. (1993) showed that in the majority of the GH_3 clonal cells studied by single cell microfluorimetry the effects of TRH on pH_i occur in two phases. The first phase is characterized by acidification that lasts only a short time and is due to Ca^{2+} mobilization from intracellular stores. The second phase consists of alkalinization that is the consequence of the activation of Na^+-H^+ antiporter. Since the results of the present study showed that the blockade of the Na^+-H^+ antiporter reduced the release of prolactin induced by TRH, it is possible that the alkalinization phase may be involved in the processes which are implicated in the release of prolactin in response to TRH.

Acknowledgements

This work was supported by a grant from the Italian National Research Council (CNR) 92.02176.CT14. We are indebted to Dr. E.J. Cragoe for the supply of amiloride analogs and to NHPP, NIDDK, NICHHD, USDA for the kind gift of a prolactin radioimmunoassay kit.

References

Blaustein, M.P., 1985, The cellular basis for cardiotonic steroid action, Trends Pharmacol. Sci. 8, 289.

Carafoli, E., 1987, Intracellular calcium homeostasis, Annu. Rev. Biochem. 56, 395.

Childs, G.V., C. Marchetti and A.M. Brown, 1987, Involvement of sodium channels and two types of calcium channels in the regulation of adrenocorticotropin release, Endocrinology 120, 2059.

Fraser, C.L. and P. Sarnacki, 1992, Regulation of plasma membrane-bound Ca^{2+}-ATPase pump by inositol phosphates in rat brain, Am. J. Physiol. 262, F411.

Frelin, C., P. Vigne, A. Ladoux and M. Lazdunski, 1988, The regulation of intracellular pH in cells from vertebrates, Eur. J. Biochem. 174, 3.

Garcia, M.L., V.F. King, J.L. Shevell, R.S. Slaughter, G. Suarez-Kurtz, R.J. Winquist and G.J. Kaczorowski, 1990, Amiloride analogs inhibit L-type calcium channels and display calcium entry blocker activity, J. Biol. Chem 265, 3763.

Hallam, T.J. and A.H. Tashjian, Jr., 1987, Thyrotropin-releasing hormone activates Na^+/H^+ exchange in rat pituitary cells, Biochem. J. 242, 411.

Kaczorowski, G.J., L.Costello, J. Dethmers, M.J. Trumble and R.L. Vendlen, 1984, Mechanisms of Ca^{2+} transport in plasma membrane vesicles prepared from cultured pituitary cells. I. Characterization of Na^+/Ca^{2+} exchange activity, J. Biol. Chem. 259, 9395.

Kaczorowski, G.J., F. Barros, J.K. Dethmers, M.J. Trumble and E.I. Cragoe, 1985, Inhibition of Na^+-Ca^{2+} exchange in pituitary plasma membrane vesicles by analogues of amiloride, Biochemistry 24, 1394.

Kolesnick, R.N., L. Musacchio, C. Thaw and M.C. Gershengorn, 1984, Thyrotropin (TSH)-releasing hormone decreases phosphatidylinositol and increases unesterified arachidonic acid in thyrotropic cells: possible early events in the stimulation of TSH secretion, Endocrinology 114, 671.

Kraicer, J. and J.W. Spence, 1981, Release of growth hormone from purified somatotrops: use of high K^+ and the ionophore A23187 to elucidate interrelations among Ca^{2+}, adenosine 3',5'-monophosphate and somatostatin, Endocrinology 108, 651.

Mariot, P., B. Dufy, M.C. Audy and P. Sartor, 1993, Biphasic changes in intracellular pH induced by thyrotropin-releasing hormone in pituitary cells, Endocrinology 132, 846.

Nachsen, D.A. and P. Drapeau, 1988, The regulation of cytosolic pH in isolated presynaptic nerve terminals from rat brain, J. Gen. Physiol. 91, 289.

Niswender, G.D., C.L. Chen, A.R. Migley, J. Meites and S. Ellis, 1969, Radioimmunoassay for prolactin, Proc. Soc. Exp. Biol. Med. 30, 793.

Repke, K.H. and W. Shonfeld, 1984, Na^+-H^+-ATPase as the digitalis receptor, Trends Pharmacol. Sci. 5, 393.

Siffert, W. and J.W.N. Akkerman, 1987, Activation of sodium -proton exchange is a prerequisite for Ca^{2+} mobilization in human platelets, Nature 325, 456.

Simchowitz, L. and E.J. Cragoe, Jr., 1986, Inhibition of chemotatic factor-activated Na^+-H^+ exchange in human neutrophils by analogs of amiloride: structure-activity relationship in the amiloride series, Mol. Pharmacol. 30, 112.

Taglialatela, M., G.F. Di Renzo and L. Annunziato, 1990, Na^+-Ca^{2+} exchange activity in central nerve endings. I. Ionic conditions that discriminate $^{45}Ca^{2+}$ uptake through the exchanger from that occurring throught voltage-operated Ca^{2+} channels, Mol. Pharmacol. 38, 385.

Tan, K.N. and A.H. Tashjian, Jr., 1984, Voltage-dependent calcium channels in pituitary cells in culture. Participation in thyrotropin releasing hormone action on prolactin release, J. Biol. Chem. 259, 427.

Tornquist, K. and A.H. Tashjian, Jr., 1989, Dual action of 1,25-dihydroxycholecalciferol on intracellular Ca^{2+} in GH_4 C_1 cells: evidence for effects on voltage-operated Ca^{2+} channels and Na^+-Ca^{2+} exchange, Endocrinology 124, 2765.

Vale, W., G. Grant, M. Amos, R. Blackwell and R. Guillemin, 1972, Culture of enzymatically dispersed anterior pituitary cells: functional validation of a method, Endocrinology 91, 562.

Vigne, P., C. Frelin, E.J. Cragoe, Jr. and M. Lazdunski, 1984, Structure-activity relationship of amiloride and certain of its analogues in relation to the blockade of the Na^+/H^+ exchange system, Mol. Pharmacol. 25, 131.