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Cholinergic regulation of Na^+-K^+-ATPase activity in rat parotid gland: changes after castration

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Abstract

In this study, we investigated the different signalling pathways involved in muscarinic acetylcholine M_3 receptor-dependent modulation of Na^+-K^+-ATPase in parotid glands from normal and castrated rats. Carbachol inhibited the enzyme activity in parotid glands from control rats while it stimulated the enzyme activity in castrated rats. The inhibition of Ca^{2+} calmodulin by trifluoperazine abolished the inhibitory effect of carbachol in control rats, while the inhibition of protein kinase C by staurosporine stimulated Na^+-K^+-ATPase. In castrated rats, trifluoperazine inhibited the carbachol-stimulant effect while staurosporine had no effect. Results indicate that in control glands the activation of a phospholipid-Ca^{2+} calmodulin-dependent protein kinase C is responsible for the inhibitory effect of carbachol on Na^+-K^+-ATPase activity. In castrated rats, the activation of the enzyme by carbachol is regulated by its Ca^{2+} calmodulin-stimulating action, and not by activation of protein kinase C. The activation of the Na^+-K^+-ATPase observed in castrated rats resulted in a decrease in carbachol-induced net K^+ efflux and thereby could decrease salivary fluid production.

Keywords: Na^+-K^+-ATPase; Parotid gland; Carbachol

1. Introduction

Epithelial cells regulate the flow of water by creating concentration gradients of osmotically active material. In salivary glands, which produce hypotonic saliva, the volume and the composition of this fluid vary with the type of stimulus used. Stimulation of cholinergic and α-adrenergic receptors results in the secretion of moderately large volumes of saliva with a characteristic electrolyte composition, while stimulation of β-adrenoceptors causes the secretion of smaller volumes of saliva with a different electrolyte composition (Martinez and Cassity, 1983). Generally, fluid secretion is regulated by the combined action of four membrane transport systems, i.e., Na^+-K^+-ATPase, Na^+-K^+-$2Cl^-$ cotransporter, Ca^{2+}-activated K^+ channels in basolateral membranes and an apical conductive pathway for Cl^-, presumably involving Ca^{2+}-activated Cl^- channels (Kurihara et al., 2000; Turner, 1993).

Na^+-K^+-ATPase is an ubiquitous membrane-bound enzyme that uses the energy released from ATP hydrolysis to maintain osmotic balance and to produce an electrochemical gradient of Na^+ and K^+ across the plasma membrane (Skou, 1988). The enzyme not only maintains the membrane potential of excitable neural and muscle cells but also is involved in reabsorption of Na^+ in the kidney (Jorgensen, 1982) and in salivary glands (Kurihara et al., 1990).

Na^+-K^+-ATPase is found in the parotid gland, along the apical and basolateral plasma membranes of acinar, striated and excretory duct cells. The apical Na^+-K^+-ATPase could participate in series with basolateral sodium and chloride entry pathways in driving secretory electrolyte fluxes (Speight and Chisholm, 1984; Conteas et al., 1986; Simson and Chao, 1994). Furthermore, it is suggested that in acinar cells Na^+ is not only transported paracellularly but is also actively transported intracellularly into the luminal space by the Na^+-K^+-ATPase located on luminal plasma membranes. Water is passively transported to the luminal space to form a plasma-like isotonic primary saliva, while in the duct cells the same ion is selectively re-absorbed intracellularly by the

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enzyme, which is found in abundance along the many infoldings of the basal plasma membranes, thus producing hypotonic saliva (Iwano et al., 1987). In addition, hormonal control of the enzyme in the granular convoluted tubule cells of the submandibular gland has been described (Kurihara et al., 1996).

In spite of the role of the Na^+-K^+-ATPase in saliva secretion, little is known about its regulation by secretagogues . Muscarinic stimulation produces copious amounts of parotid saliva, equivalent to ~5% of the whole body water content/h in mice (Evans et al., 1999). Taken together the role of Na^+-K^+-ATPase in saliva secretion and the effect of muscarinic stimulation of the parotid gland, the aim of the present work was to study whether the activation of the M_3 muscarinic acetylcholine receptor regulates Na^+-K^+-ATPase activity. Moreover, the activity of this enzyme in granular convoluted tubule cells of the submandibular gland is under multihormonal control (Sims-Sampson et al., 1984) and results obtained in our laboratory showed changes in parotid function resulting from castration (Busch and Borda, 2002; 2003). We therefore also studied the influence of castration on the regulatory effect of carbachol on Na^+-K^+-ATPase activity.

2. Methods

2.1. Animals

Male Wistar rats weighing 250–300 g were used throughout. Animals had free access to food and water until the night before experiments, when food, but not water, was withdrawn. The following experimental groups were used: (a) control animals (normal); (b) castrated animals; and (c) castrated testosterone-treated animals. Castration was performed under ether anesthesia by removing the testicles through bilateral cuts in the scrotum 21 days before experiments, and testosterone replacement treatment of castrated rats was carried out by injecting testosterone (1 mg 100 g body weight^{-1}) in 0.20 ml absolute ethanol s.c., beginning at 15 days after castration and daily thereafter for 7 days (Busch and Borda, 2002). Control castrated rats received 0.20-ml absolute ethanol. The success of castration was evident from the significant reduction of testosterone levels, as observed in Table 1. Animals were cared according to "The Guide to the Care and Use of Experimental Animals" (DHEW Publication, NIH 80-23).

2.2. Serum testosterone levels

After the parotid glands were removed under light ether anesthesia, the animals were killed by cardiac puncture. Blood from the left ventricle was collected, centrifuged and stored in Eppendorf tubes at −70 °C. Serum testosterone levels were determined by a fluoroimmunological method (FIA-Delfia) (Hemmila et al., 1984). Minimum sensitivity of the kit was 0.2 nmol l^{-1}.

Table 1
Testosterone serum levels (nmol l^{-1}) in control, castrated and castrated testosterone (To)-treated rats obtained 21 days after castration

Group	Control	Castrated	Castrated To-treated
Testosterone levels	18 ± 2.0[a]	0.3 ± 0.05[b]	25 ± 2.8

Testosterone levels from 15 animals of each group were determined by a fluoroimmunological technique as described in Methods.

[a] Significant difference from castrated ($P<0.001$) and castrated testosterone-treated rats ($P<0.05$).

[b] Significant difference from control and castrated testosterone-treated rats ($P<0.001$).

2.3. Membrane preparation for ATPase activity determination

Free connective tissue and fat were gently remove from the parotid glands under a magnifying glass, and the anterior lobe was cut into small slices which were placed in tubes containing 500 μl of Krebs–Ringer bicarbonate solution pH 7.4 bubbled with 95% O_2 and 5% CO_2 and incubated at 37 °C for 30 min. When used, inhibitors were included from the beginning of the incubation time and carbachol was added in the last 20 min. The reaction was stopped by removing the parotid slices and homogenizing them at 4 °C in tubes containing 750 μl of Tris–HCl 10 mM, EDTA 1 mM, LiBr 0.4 M (hypotonic shock) and supplemented with the protease inhibitor, phenylmethylsulfonyl fluoride 0.1 mM. The homogenates were centrifuged for 10 min at $1000 \times g$, and the supernatants were collected and spun down for 20 min at $9000 \times g$. Both steps almost completely eliminated nucleus, mitochondrial and lisosomal fractions, and the resultant supernatant was centrifuged for 60 min at $100{,}000 \times g$. The pellet was then re-suspended in Tris–HCl 10 mM, EDTA 1 mM and the same protease inhibitor and stored at −70 °C until used.

2.4. Determination of NA^+-K^+-ATPase activity

Membrane aliquots (approximately 10–20 μg of protein) were transferred to the Na^+-K^+-ATPase assay medium (final volume 172 μl) containing in mM: NaCl, 100; KCl, 20; $MgCl_2$, 3; Tris–HCl, 160 (pH 7.4) and Na_2ATP, 4 and incubated for 30 min at 37 °C in the absence or presence of 2×10^{-3} M ouabain. When ouabain was present, NaCl and KCl were omitted from the incubation medium and replaced by Tris–HCl. The reaction was stopped by the addition of 40 μl of cold trichloroacetic acid 30%. Samples were centrifuged at $3000 \times g$ for 10 min and the inorganic phosphate liberated (total ATPase activity) was measured. Na^+-K^+-ATPase activity was calculated as the difference between the means of the total ATPase activity and the ouabain-sensitive ATPase activity

and expressed as μmol Pi mg protein^{-1} h^{-1} (Calvino et al., 2002).

2.5. [^{3}H]ouabain binding assay

Parotid glands were excised and immediately homogenized at 4 °C in 6 to 8 volumes of Tris–HCl 10 mM buffer pH 7.4, 0.25 M sucrose, 5 mM $MgCl_2$ and supplemented with protease inhibitors (0.1 mM phenylmethyl-sulfonyl fluoride, 1 mM sodium ethylenediaminetetra-acetate, 2 μg ml^{-1} leupeptin, 2 μM pepstatin A, 1 μg ml^{-1}aprotinin and 1 mM iodoacetamide). The homogenate was centrifuged as described above to obtain a plasma membrane-enriched fraction. For binding assay, the plasma membrane-enriched fraction was re-suspended in the same Tris buffer described above (without sucrose) at a concentration of ~500 μg ml^{-1} of protein. The saturation assay was performed by incubation of 200–300 μg of protein with 5 to 2560 nM of [^{3}H]ouabain ([^{3}H]ouabain, NEN™ Life Science Products, Boston, MA, USA) in a total volume of 150 μl for 1 h at 37 °C with continuous shaking. Binding was stopped by adding 2 ml of ice-cold buffer, followed by rapid filtration (Whatman GF/c). Filters were rinsed with 12 ml of ice-cold buffer, transferred into vials containing 10 ml of scintillation cocktail and counted in a liquid scintillation spectrometer. Nonspecific binding was determined in the presence of 2 μM ouabain and never exceeded 10% of the total binding. Radioactivity bound was lower than 10% of total counts.

2.6. Net K^+ efflux determination

Parotid slices prepared as described above were incubated in 1 ml of oxygenated Krebs–Ringer bicarbonate buffer at 37 °C in the presence or absence of carbachol 10^{-5} M, ouabain 5×10^{-4} M and carbachol 10^{-5} M plus ouabain 5×10^{-4} M or 4-diphenylacetoxi-*N*-methylpiperidine methiodide (4-DAMP) 10^{-5} M. Aliquots of 200 μl of the medium were removed at 2, 5 and 10 min. At the end of the incubation period (10 min), the slices were homogenized in the remaining Krebs–Ringer bicarbonate medium and the K^+ concentration in the homogenate and in the aliquots removed in the course of the experiments was measured in triplicate in an Instrumentation Laboratories flame photometer. Net K^+ efflux is presented as the percentage of the K^+ present in the slices.

2.7. Drugs

Carbachol, ouabain, staurosporine, trifluoperazine, Ca^{2+} ionophore A 23187, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. U-73122 and 4-DAMP were from RBI. The drugs were diluted in the bath to achieve the final concentration stated in the text.

2.8. Data analysis

Student's *t*-test for unpaired values was used to determine the levels of significance. When multiple comparisons were necessary after analysis of variance, the Student–Newman–Keuls multiple comparison test was applied. Differences between means were considered significant if $P < 0.05$.

3. Results

3.1. Characterization of [^{3}H]ouabain binding sites in membranes from control, castrated and castrated testosterone-treated rats

We studied the expression of the enzyme Na^+-K^+-ATPase and its changes after castration by means of [^{3}H]ouabain binding assay. We observed the presence of two ouabain binding sites with high and low affinity, respectively (Fig. 1, upper and lower panel). In both sites, [^{3}H]ouabain bound specifically to parotid membranes and the binding became saturated with increasing concentrations of the ligand. In saturation studies (Fig. 1A, upper and lower panel) and Scatchard analysis (Fig. 1B, upper and lower panel), an irreversible interaction was established. Castration modified [^{3}H]ouabain binding. Thus, parotid glands from castrated rats showed a decrease in the number of low-affinity [^{3}H]ouabain binding sites (B_{max}) and an increase in the affinity (K_d) of both high- and low-affinity sites. Table 2 shows a decrease of 43% of the B_{max} (low-affinity sites) and a decrease of 80% and 58% of the K_d values for low- and high-affinity sites, respectively, in castrated rats, and the reversal of this with testosterone treatment, which restored the [^{3}H]ouabain-binding parameters to control values.

3.2. Evaluation of the activity of NA^+-K^+-ATPase in parotid glands from control, castrated and castrated testosterone-treated rats

To determine whether the changes observed in B_{max} and K_d of [^{3}H]ouabain in castrated rats resulted in changes in enzyme function, we evaluated basal and carbachol-stimulated Na^+-K^+-ATPase activity in parotid membranes from control, castrated and castrated testosterone-treated rats. We observed that under basal conditions the activity of Na^+-K^+-ATPase (μmol Pi mg protein^{-1} h^{-1}) was similar in the three groups; i.e. controls 2.5 ± 0.30; castrated 2.6 ± 0.24 and castrated testosterone-treated rats 2.7 ± 0.31 ($n = 6$ experiments/group).

The ability of carbachol to induce changes in the activity of Na^+-K^+-ATPase is shown in Fig. 2. Over the range from 10^{-7} to 10^{-5} M, carbachol exerted an inhibitory effect in parotid glands from control rats and a stimulatory effect in parotid glands from castrated ones. In testosterone-treated castrated rats, the stimulatory effect of carbachol at 10^{-6}

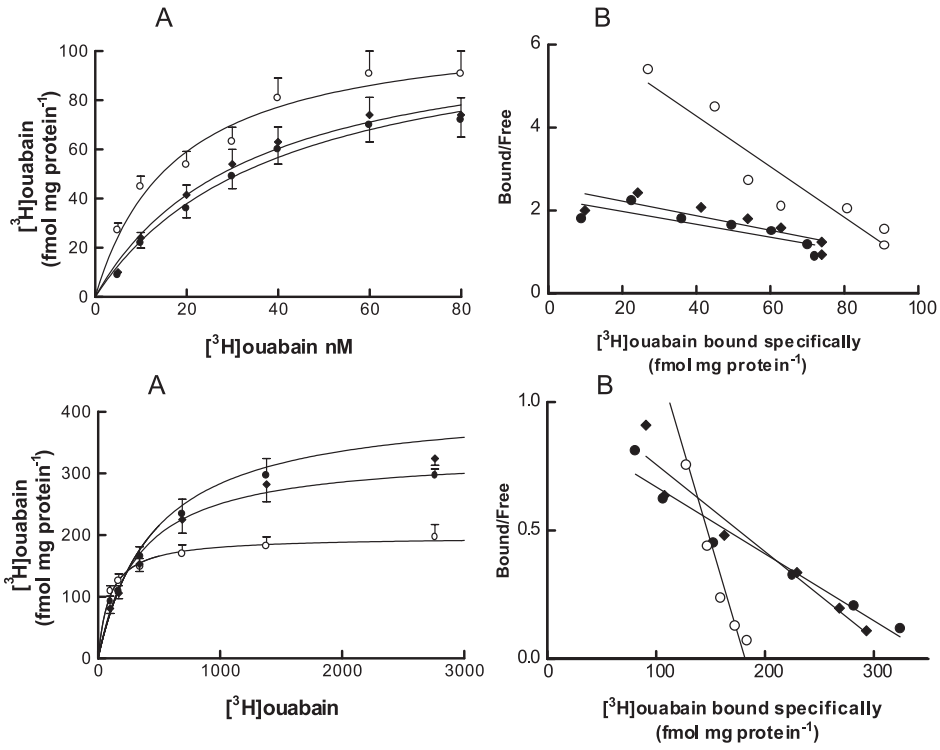

Fig. 1. Saturation curves (A) and Scatchard plots (B) of parotid gland membranes from control (●), castrated (○) and castrated testosterone-treated (♦) rats incubated with different concentrations of [^{3}H]ouabain. The figure shows high-affinity (upper panel) and low-affinity (lower panel) binding parameters. Each point represents the mean ± S.E.M. of four experiments performed in duplicate.

and 10^{-5} M was reversed while at 10^{-7} M it still showed the stimulatory action.

3.3. Receptor and mechanism involved in carbachol regulatory effect on the NA^+K^+ pump

To assess whether the M_3 muscarinic acetylcholine receptor subtype was involved in the carbachol regulatory action, parotid gland tissue was incubated with the selective antagonist of the M_3 subtype, 4-DAMP (10^{-7} M). Table 3 shows that 4-DAMP inhibited both carbachol actions: the stimulation of enzyme activity in parotid glands from castrated rats and the inhibition of enzyme activity in control and castrated testosterone-treated rats.

To determine the mechanism by which carbachol had a modulatory action on Na^+-K^+-ATPase activity, parotid gland tissue from normal and castrated rats was incubated with

Table 2
Binding of [^{3}H]ouabain to parotid membranes from control, castrated and castrated testosterone (To)-treated rats

Group	K_d (nM)		B_{max} (fmol mg protein^{-1})	
	Low	High	Low	High
Control	457.9 ± 9.6	39.5 ± 7.1	413.3 ± 8.2	112.8 ± 9.6
Castrated	94.2 ± 12.2[a]	17.7 ± 3.8[b]	197.2 ± 5.1[a]	111.4 ± 8.0
Castrated To-treated	328.5 ± 29.4	33.3 ± 6.1	332.9 ± 10.0	110.6 ± 8.7

The membranes from parotid glands were prepared for binding assays as referred to in Methods. B_{max} and K_d were calculated from saturation curves and analysed with nonlinear regression and Scatchard plot. Values indicate means ± S.E.M. of four experiments run in duplicate.

[a] Significantly different from control and castrated To-treated rats ($P<0.001$).

[b] Significantly different from control ($P<0.05$).

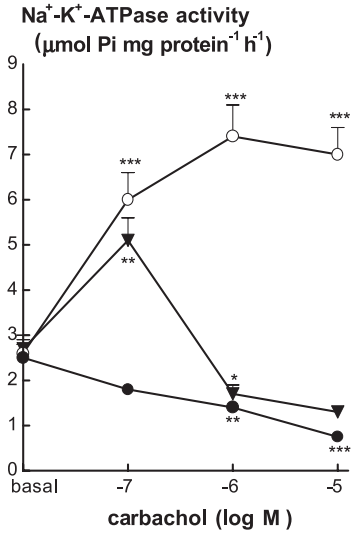

Fig. 2. Effect of carbachol on Na^+-K^+-ATPase activity in parotid glands from control (●), castrated (○) and castrated testosterone-treated (▼) rats. Each point represents the mean ± S.E.M. of six independent experiments performed in duplicate. *$P<0.05$; **$P<0.01$; ***$P<0.001$ vs. basal values.

Table 3
Effect of different inhibitor agents on the regulatory effect of carbachol on Na^+-K^+-ATPase activity in parotid glands from control, castrated and castrated testosterone (To)-treated rats

Additions	Control	Castrated	Castrated To-treated
None (A)	2.5 ± 0.30^a	2.6 ± 0.24^b	2.7 ± 0.31^a
Carbachol 10^{-6} M (B)	1.4 ± 0.12^c	7.4 ± 0.60^d	1.7 ± 0.20^e
Carbachol 10^{-6} M + 4-DAMP 0^{-7} M (C)	2.7 ± 0.25	3.6 ± 0.40	2.5 ± 0.20
Carbachol 10^{-6} M + U-73122 5×10^{-6} M (D)	2.8 ± 0.30	4.6 ± 0.40^a	2.7 ± 0.24
Carbachol 10^{-6} M + TFP 5×10^{-6} M (E)	2.5 ± 0.23	4.3 ± 0.35^a	2.5 ± 0.24
Carbachol 10^{-6} M + Staurosporine 10^{-9} M (F)	4.1 ± 0.40^c	7.1 ± 0.70^f	4.5 ± 0.30^c

Parotid slices were incubated in the presence of the inhibitors and carbachol as stated in Methods and then membranes were prepared for the ATPase activity determinations. Data are the means ± S.E.M. of six experiments of each group. Each column was analysed independently.

[a] Significant difference from B and F.
[b] Significant difference from all other groups.
[c] Significant difference from all other groups except vs. C.
[d] Significant difference from all other groups except vs. F.
[e] Significant difference from groups except vs. B.
[f] Significant difference from all other groups A, D and F.

different inhibitors of the enzymatic pathways involved in M_3 activation. Table 3 shows that inhibition of phospholipase C by U-73122 (5×10^{-6} M) resulted in a blockade of the inhibitory effect and an attenuation of the stimulatory effect of the agonist in control and castrated rats respectively. Moreover, the inhibition of Ca^{2+} calmodulin by trifluoperazine (5×10^{-6} M) significantly inhibited the action of carbachol on Na^+-K^+-ATPase in control and castrated animals. In contrast, the blockade of protein kinase C by staurosporine (10^{-9} M) reversed the carbachol inhibitory action in control rats while it had no effect in castrated ones. Even more, staurosporine significantly reversed the concentration-dependent carbachol inhibitory action in control rats ($P < 0.001$) and this effect was blunted by trifluoperazine ($P < 0.001$) (Fig. 3). In parotid glands from castrated testosterone-treated rats, the agonist and the enzymatic inhibitors had the same effect as in control group (Table 3).

3.4. Effect of phorbol esters and Ca^{2+} ionophore on NA^+-K^+-ATPase activity

In order to elucidate whether the activation of protein kinase C and/or the increase in intracellular Ca^{2+} concentration were involved in the differences observed in the regulatory action of carbachol on Na^+-K^+-ATPase activity in parotid gland from control and castrated rats, we studied the effect of PMA and A 23187. Fig. 4A shows that the exogenous activation of protein kinase C with PMA (10^{-6} M) resulted in a decrease in basal enzyme activity

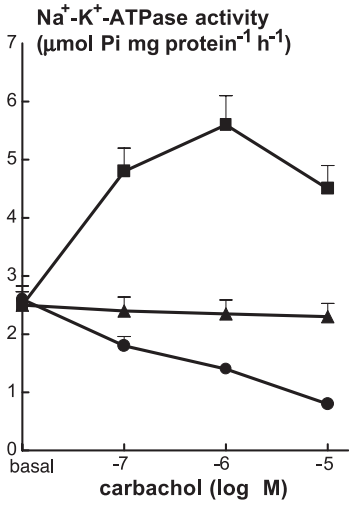

Fig. 3. Effect of carbachol in the absence (●) or the presence of staurosporine 10^{-9} M (■) and staurosporine 10^{-9} M plus trifluoperazine 5×10^{-6} M (▲) on Na^+-K^+-ATPase activity in parotid glands from control rats. Each point represents the mean ± S.E.M. of four independent experiments performed in duplicate.

in both groups. A non-ionic detergent-like effect was excluded by the lack of effect of an inactive phorbol (data not shown). The Ca^{2+} ionophore A 23187 (10^{-7} M) induced activation of Na^+-K^+-ATPase in both control and castrated rats. The stimulatory effect of the ionophore was blocked by trifluoperazine 5×10^{-6} M ($P < 0.01$) (Fig. 4B). Results demonstrated that direct activation of protein kinase

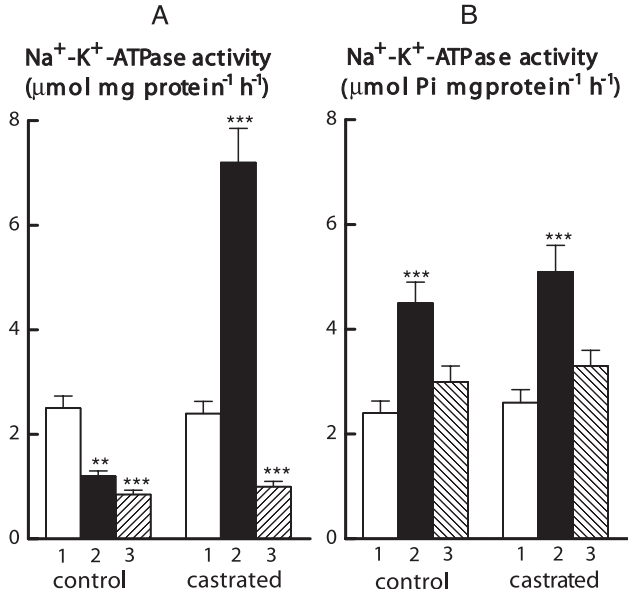

Fig. 4. Panel A: Effect of phorbol ester (PMA) on Na^+-K^+-ATPase activity in parotid glands from control and castrated rats. (1) Basal; (2) carbachol 10^{-6} M; (3) PMA 10^{-6} M. Panel B: Effect of Ca^{2+} ionophore A 23187 on Na^+-K^+-ATPase activity in parotid glands from control and castrated rats. (1) Basal; (2) A 23187 10^{-6} M; (3) A 23187 10^{-6} M plus trifluoperazine 5×10^{-6} M. Each bar represents the mean ± S.E.M. of four independent experiments performed in duplicate. $^{**}P < 0.01$; $^{***}P < 0.001$.

C or an increase in intracellular Ca^{2+} concentration resulted in inhibition or activation of the enzyme in both control and castrated animals.

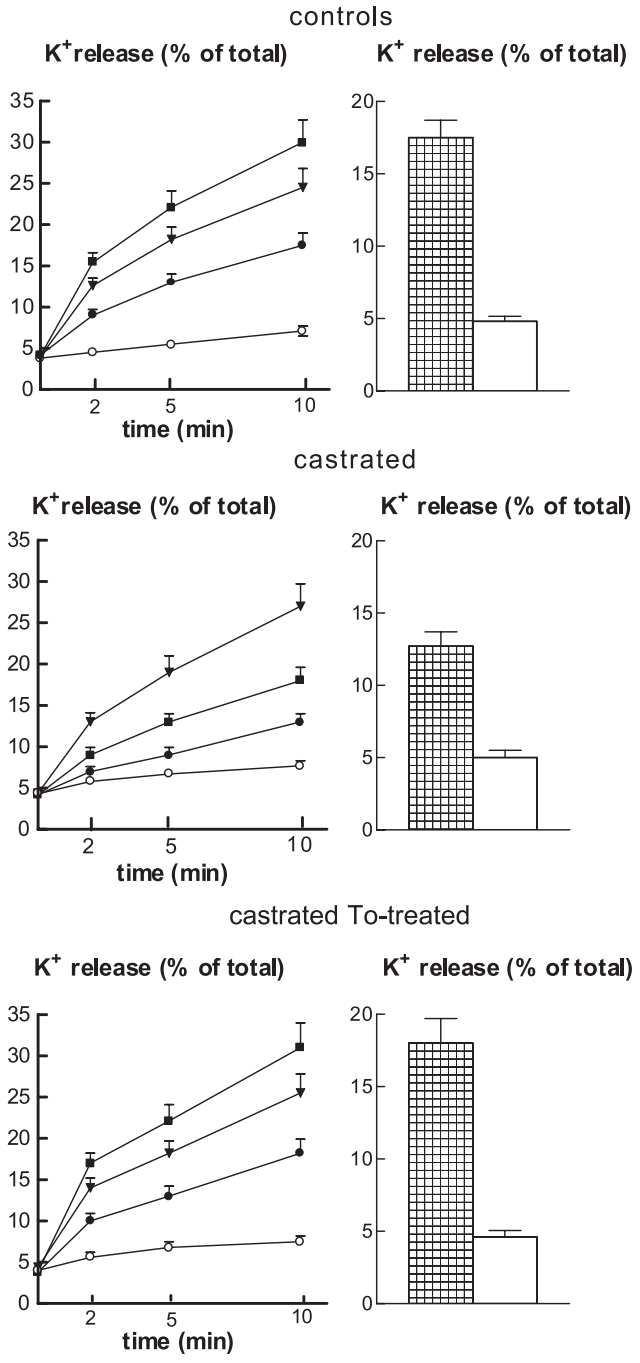

Fig. 5. Panel A: Extent of net K^+ efflux from the parotid slices from control (top), castrated (middle) and castrated testosterone-treated (bottom) rats under basal conditions (○) and after the addition of ouabain 5×10^{-4} M (▼), carbachol 10^{-5} M (●) and ouabain 5×10^{-4} M plus carbachol 10^{-5} M (■). Each point represents the mean ± S.E.M. of 4 independent experiments. Panel B: Effect of carbachol alone (⊞) and in the presence of 4-DAMP (□) on K^+ release from parotid slices from control, castrated and castrated testosterone-treated rats. Each bar represents the mean ± S.E.M of four independent experiments.

3.5. Effect of carbachol on net K^+ efflux from parotid glands from control, castrated and castrated testosterone-treated rats

Since the net efflux of K^+ from salivary glands appears to be the result of two simultaneous and opposing mechanisms, namely, passive K^+ efflux induced by secretagogues and active K^+ re-uptake by the Na^+/K^+ pump, we investigated whether the alteration of the carbachol effect on the Na^+/K^+ pump observed in castrated rats resulted in an alteration of net K^+ efflux from parotid slices. Fig. 5A shows that the basal net K^+ efflux was not altered by castration. Carbachol (10^{-5} M) induced a significant increase in net K^+ efflux in the three groups under study but, as expected, the increase was less ($P<0.01$) in the slices from castrated rats. Ouabain (5×10^{-4} M) increased the basal net K^+ efflux in the three groups under study, indicating that an ouabain-sensitive Na^+-K^+-ATPase is responsible for the K^+ re-uptake during secretion (Fig. 5A). The combination of carbachol 10^{-5} M and ouabain 5×10^{-4} M resulted in synergism in control and castrated testosterone-treated rats but antagonism was observed in castrated rats (Fig. 5A). The muscarinic acetylcholine M_3 receptor subtype antagonist, 4-DAMP, inhibited the effect of carbachol on net K^+ efflux in the three groups studied (Fig. 5B).

4. Discussion

The present data indicate that Na^+-K^+-ATPase in rat parotid gland is inhibited by carbachol through a phospholipid-Ca^{2+}-calmodulin-dependent protein kinase C. Castration induced changes in the expression and affinity of [^{3}H]ouabain binding and alterations in the muscarinic post-receptor pathway, which resulted in an opposite effect of carbachol.

The inhibitory effect of carbachol observed in control rats was abolished when phospholipase C and Ca^{2+} calmodulin activities were blocked, restoring Na^+-K^+-pump activity to basal values. However when protein kinase C was inhibited, Na^+-K^+-pump activity increased, an effect that was prevented by inhibition of Ca^{2+} calmodulin. These results suggest that protein kinase C is involved in the inhibitory action of carbachol. It is likely that in parotid glands carbachol induced both effects, stimulation and inhibition, through the activation of Ca^{2+} calmodulin and protein kinase C, respectively. Thus, when protein kinase C activity was blocked, the stimulatory effect mediated by Ca^{2+} calmodulin was unmasked. The fact that trifluoperazine antagonized the inhibitory effect of carbachol indicates that a Ca^{2+} calmodulin-dependent protein kinase C is responsible for this inhibitory effect. As a matter of fact, a protein kinase C-α was described as the principal Ca^{2+}-dependent isoform associated with cholinergic stimulation in parotid cells (Terzian and Rubin, 1993). Results obtained

with renal proximal tubule cells from rats suggest that the Na^+-K^+-ATPase is an effector protein for protein kinase C and that dopamine inhibition of enzyme activity is mediated by this kinase (Bertorello and Aperia, 1988). However, there is evidence for the participation of Ca^{2+} calmodulin in the α-adrenergic stimulation of Na^+-K^+-ATPase activity in rat renal tubule cells (Aperia et al., 1992), and in the Ca^{2+} stimulatory action in rat brain homogenates (Powis, 1985).

Our results obtained with phorbol ester (PMA) and the Ca^{2+} ionophore (A 23187) are in concordance with the above reports. In control rats, the direct activation of protein kinase C with PMA decreased Na^+-K^+-ATPase basal activity, while the Ca^{2+} ionophore increased Na^+-K^+-pump activity, suggesting that the increase in intracellular Ca^{2+} concentration activates Ca^{2+} calmodulin which in turn stimulates Na^+-K^+-ATPase activity. Given that the increase in intracellular Ca^{2+} concentration elicited by carbachol activates potassium and chloride channels (Petersen, 1992), and has the opposite effect on net K^+ efflux by the Na^+-K^+-ATPase (Martinez and Camden, 1983), it is plausible to think that cholinergic agonists abolish the effect of the enzyme through activation of protein kinase C.

Two binding sites for [^{3}H]ouabain were observed in parotid glands, with high and low affinity, respectively. Castration induced an increase in the affinity of both high- and low-affinity binding sites and a decrease in the number of low-affinity binding sites for [^{3}H]ouabain in parotid glands. These effects were restored by testosterone treatment. The Na^+-K^+-pump is present in acinus cells as well as in duct cells in parotid glands (Iwano et al., 1987). Since orchiectomy affects the structure of the acinus as well as the structure of intercalated and striated ducts (Jezek et al., 1996), the alteration in Na^+-K^+-ATPase activity probably involved the enzyme present in both structures (acinus and duct cells). The fact that only low-affinity binding sites were down-regulated in castrated rats, in addition with an increase of affinity, resulted in an unchanged basal Na^+-K^+-ATPase activity in this group. Kurihara et al. (1996) described that the Na^+-K^+-ATPase α subunit of rat submandibular gland is regulated by steroid hormones. Changes induced by castration in this catalytic subunit could result in changes in [^{3}H]ouabain affinity.

In contrast with the effect observed in parotid glands from control rats, carbachol increased Na^+-K^+-ATPase activity in castrated rats. This activation was mimicked by a Ca^{2+} ionophore and was inhibited by U-73122 and trifluoperazine, indicating that phospholipase C and Ca^{2+} calmodulin were involved in this carbachol effect. Ca^{2+} calmodulin is known to be involved in the activation of Na^+-K^+-ATPase in renal tubules (Aperia et al., 1992) and brain (Powis, 1985). Thus, it is likely that in parotid glands from castrated rats Ca^{2+} calmodulin constituted a positive signal for the Na^+-K^+ pump, as was observed in controls. In contrast, protein kinase C activity seems not to be involved in the pathway triggered by carbachol in castrated rats. Considering the lack of effect of staurosporine on carbachol action together with the ability of PMA to decrease basal enzyme activity, we could suggest that even though protein kinase C preserved the ability to inhibit the pump, in castrated rats carbachol failed to activate the kinase. Other evidence supporting the view that castration induced an alteration in the carbachol pathway comes from the effect of testosterone replacement. In this group, low concentrations of carbachol induced activation of the pump, while at higher concentrations inhibition was observed. This result suggests that a higher concentration of carbachol was needed to achieve activation of protein kinase C in castrated testosterone-treated rats.

A diagram to tie together the various systems studied and proposing a model for the mechanism by which carbachol inhibits or stimulates Na^+-K^+-ATPase activity in parotid slices from control and castrated rats is shown in Fig. 6.

In order to explore whether alteration in the modulatory action of carbachol on Na^+-K^+-ATPase activity resulted in alteration of ionic fluxes which play a key role in fluid secretion (Putney, 1976), we studied the effect of castration on net K^+ efflux. Basal net K^+ efflux did not change after castration but, as expected, net K^+ efflux after carbachol stimulation was diminished. The magnitude of net K^+ efflux, after carbachol stimulation, is in the ratio of two opposing mechanisms, a loss via a Ca^{2+}-activated K^+ channel (Lee and Turner, 1993) and a gain through Na^+-K^+-ATPase activity (Martinez and Quissell, 1976). Thus, in castrated rats the activation of the pump induced by carbachol resulted in an increase in K^+ uptake and a decrease in net K^+ efflux. The participation of the Na^+-K^+-ATPase was

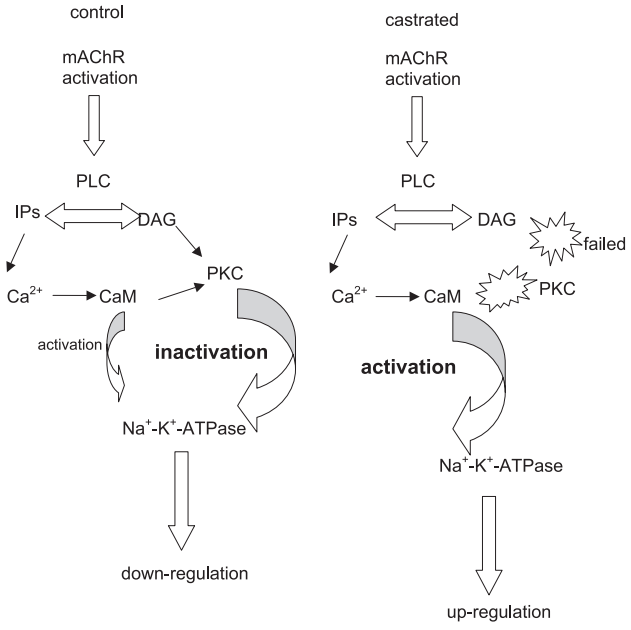

Fig. 6. Scheme illustrating hypothetical role of Ca^{2+} calmodulin and protein kinase C mediated effects with opposing physiological actions in the regulation of Na^+-K^+-ATPase activity. mAChR: muscarinic acetylcholine receptor; PLC: phospholipase C; IPs: phosphoinositides; DAG: diacylglycerol; PKC: protein kinase C; CaM: Ca^{2+} calmodulin.

evident by the increase in basal net K^+ efflux observed in the presence of ouabain in the three groups under study. When carbachol-stimulated K^+ efflux was evaluated in the presence of ouabain, synergism was observed in control and castrated testosterone-treated rats and antagonism in castrated animals. This result was a key to find out how castration altered salivary physiology. The decrease in net K^+ efflux observed in castrated rats could be related with xerostomia associated with ageing. Although studies have determined that salivary gland function is well preserved in the healthy geriatric population and that xerostomia is a condition of systemic or extrinsic origin (Astor et al., 1999), an alteration in the glands as a result of the decrease in testosterone levels cannot be disregarded. With this respect, it is well known that salivary glands are target organs for androgens and that testosterone treatment improves Sjögren's syndrome manifestations included xerostomia (Sato and Sullivan, 1994; Vendramini et al., 1991).

Since testosterone replacement reversed the effect of castration, our results are in concordance with the regulation by testosterone of the expression and affinity of Na^+-K^+-ATPase in male rat parotid glands and its involvement in the regulatory mechanism induced by carbachol.

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