THE CALCIUM IONOPHORE A 23 187 AS A PROBE FOR STUDYING THE ROLE OF CA^{2+} IONS IN THE MEDIATION OF CARBACHOL EFFECTS ON RAT SALIVARY GLANDS: PROTEIN SECRETION AND METABOLISM OF PHOSPHOLIPIDS AND GLYCOGEN

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1. Introduction

Protein secretion by rat submaxillary or parotid glands is regulated by sympathetic and parasympathetic systems. Cholinergic agonists induce protein discharge in submaxillary and parotid glands [1–3]. Parasympathetic mediators trigger glycogen breakdown in rat submaxillary glands, as described in a previous paper [4]. Carbachol stimulates phosphate turnover in phospholipids (phospholipidic effect) in cholinoceptive glands [1,5,6]. All these cholinergic effects are calcium dependent. The protein discharge induced by carbachol is completely abolished in calcium-free medium, whereas the phospholipidic effect is only diminished by about fifty per cent [7]. The cholinergic agonist does not increase the cAMP level in salivary glands [8], on the other hand it stimulates Ca^{2+} uptake by rat pancreas and parotid glands [9,10] and rises the cGMP level in rat submaxillary glands [11]. The latter effect however seems also to be calcium dependent [11]. According to these observations calcium plays a fundamental role in all secretory processes regulated by cholinergic mediators. In the present paper we report that ionophore A 23 187 mimics carbachol effects on protein discharge and glycogen breakdown but does not trigger the phospholipidic effect. As we described in a previous paper [4] we suggest that cholinergic agonists induce protein discharge and glycogen breakdown by increasing calcium influx into secretory cells and rising the intracellular free Ca^{2+} level. On the other hand, parasympathetic mediators trigger the phospholipidic effect through a system where both the agonist and calcium seem to be required at the membrane level, since, A 23 187, which introduces Ca^{2+} into the cell, does not by itself mimic the cholinergic response on the phospholipidic effect.

2. Materials and methods

[U-^{14}C]leucine (297 mCi/mM), [U-^{14}C]glucose (150 mCi/mM), [^{32}P]phosphate (2 mCi/ml) were obtained from CEA, Saclay (France). Carbachol was purchases from Mann Research Laboratory, New York (USA). Atropine was obtained from Calbiochem (Los Angeles, USA), theophylline from Merck-Darmstadt (Germany), dibutyryl-cyclic GMP and cyclic GMP from Boehringer Mannheim (Germany). A 23 187 was a kind gift from Eli-Lilly and Co. (Indianapolis, Ind., USA). The ionophore was dissolved in ethanol to a concentration of 1 mg/ml. The final concentration of the incubation medium was 1 μM A 231 87 and 0.7% ethanol (at this concentration the solvent did not affect the protein discharge or the metabolism).

Glucostat was purchased from Worthington Biochemical Corp. (USA).

Male albino rats (6–7 weeks, 150–200 g) were killed by decapitation. The submaxillary and parotid glands were immediately removed and cut into small pieces (2–3 mm in size).

The incubations were performed in a Warburg incubator. Each incubation vessel contained 100–150

mg glands in 7 ml of Krebs–Ringer bicarbonate buffer with 0.55 mM glucose at 37°C, equilibrated with 95% O_2, 5% CO_2 gas.

2.1. *Glycogen measurement*

Rat submaxillary glands were incubated in Krebs Ringer buffer with [U-^{14}C]glucose during 60 min. At the end of incubation time, the glands were blotted, weighed and digested in 30% KOH (60 min. at 100°C). Glycogen was precipitated by ethanol (70% final concentration), washed twice by 70% ethanol. The sediment was dissolved and the proteins were eliminated by 10% TCA; glycogen was again precipitated by 70% ethanol.

After glycogen hydrolysis (2 N HCl, 2 hr, 100°C) glucose was determined by the glucose oxidase method [12].

2.2. *Protein secretion*

The protein discharge was determined as previously described [4] except that the proteins were labelled with [U-^{14}C]leucine. The secretion is indicated by expressing the amount of labelled proteins present in the incubation medium as a percentage of the sum of labelled proteins in tissue and medium.

2.3. *Phospholipid labelling*

Parotid glands were preincubated with [^{32}P]phosphate during 30 min according to Hokin [5]. 2 μM carbachol or 1 μM ionophore A 23 187 were then added an the incubation was pursued during various times. At the end of the incubation period glands were blotted, weighed and homogenized with ice-cold 10% TCA. The homogenate was centrifuged and phospholipids were extracted from the pellet by the solvent (ethanol, ether, chloroform v/v/v, 40:40:20) 3-fold during 30 min. The radioactivity of the extracts was measured, as described below.

2.4. *Protein phosphorylation*

Parotid glands were incubated with [^{32}P]phosphate as described in phospholipid labelling.

Method 1 – Glands were homogenized in ice-cold 10% TCA and centrifuged.

Method 2 – Glands were homogenized in ice-cold 0.15 M sodium phosphate buffer, pH 6.4. The homogenate was centrifuged at 34 000 *g* during 30 min and the proteins from the supernatant were precipitated by 10% TCA.

Phospholipids were extracted from the TCA pellets obtained by Methods 1 and 2, as described above. The protein sediment was then suspended in 5% TCA, heated at 90°C for 15 min. After centrifugation, the proteins were solubilized in 0.5 N NaOH and an aliquot was counted.

2.5. *Radioactivity measurements*

The radioactivity of each sample (protein, phospholipid extract and glycogen) was counted on a Nuclear Chicago model C 115 low background counter as previously described [4].

3. Results

3.1. *Protein secretion*

Fig. 1 demonstrates that 2 μM carbachol and 1 μM ionophore A 23 187 induce protein discharge in rat parotid glands. The ionophore seems to be as efficient as carbachol to promote the protein output. Since Schultz et al. [11] reported that acetylcholine rises

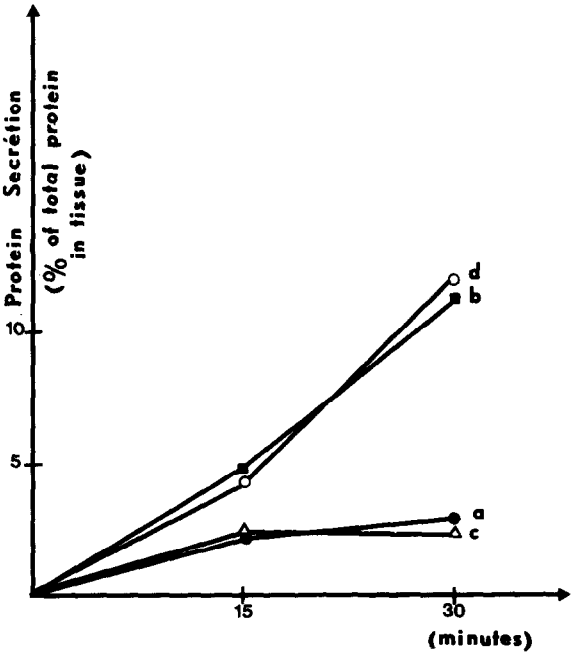

Fig. 1. Discharge of labeled proteins into the incubation medium following pulse labeling experiments. (a) Control; (b) 2 μM carbachol; (c) 2 μM carbachol + 10 μM atropine; (d) 1 μM A 23 187.

Table 1
Effects of cGMP, cGMP-DB and carbachol on labeled proteins discharged into the incubation medium (expressed as percent of total proteins in tissue), following pulse labeling experiments

	Control	cGMP		cGMP-DB		Carbachol
		1 μM	100 μM	1 μM	100 μM	2 μM
Theophylline (100 μM)						
–	2.8	2.9	3.6	3.2	2.8	11
+	3.1	2.6	3.7	3.1	4	–

Discharge time: 30 min.

cGMP level, we investigated the effect of cGMP on protein discharge. Table 1 shows that cGMP or $cGMP_{DB}$ at different concentrations, with or without theophylline do not stimulate significantly the secretion by parotid glands. On the other hand, it has been confirmed (results not shown) that cAMP stimulates glycoprotein secretion in rat submaxillary glands, which agrees with the data of Babad et al. [13]. However the cAMP effect concerns the adrenergic regulation of secretory processes and not the cholinergic one [4,8].

3.2. *Glycogen metabolism*

Two μM carbachol triggers glycogen breakdown in rat submaxillary glands (fig. 2) (in parotid gland there is no glycogen storage). In glands incubated without mediator during 60 min the glycogen level is 370 μg/g tissue. About 85% of this glycogen breaks down when glands are incubated with cholinergic agonists. This carbachol effect is abolished by atropine. The ionophore A 23 187 also evokes a glycogen breakdown. As shown in fig. 3 carbachol or the ionophore reduce the [^{14}C]glucose incorporation into glycogen.

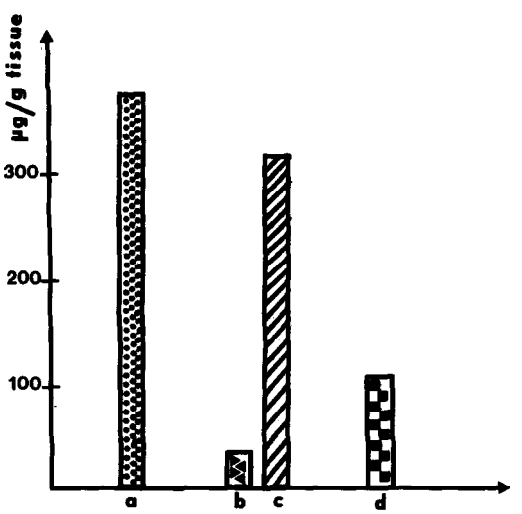

Fig. 2. Effect of carbachol and A 23 187 om glycogen levels in rat submaxillary glands incubated during 60 min. (a) Control; (b) 2 μM carbachol; (c) 2 μM carbachol + 10 μM atropine; (d) 1μM A 23 187.

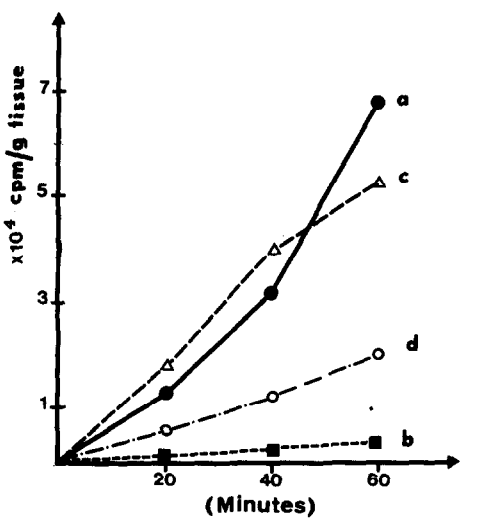

Fig. 3. Effect of carbachol and A 23 187 on [^{14}C]glucose incorporation into glycogen. (a) Control; (b) 2 μM carbachol; (c) 2 μM carbachol + 10 μM atropine; (d) 1 μM A 23 187.

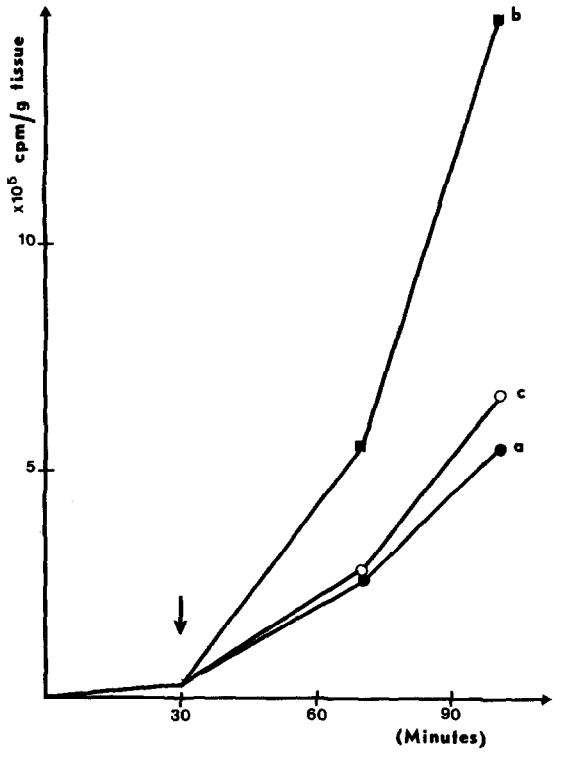

Fig. 4. Effect of carbachol and A 23 187 on [^{32}P]phosphate incorporation into total phospholipids. Carbachol or ionophore were added at the time indicated by the arrow. (a) Control; (b) 2 μM carbachol; (c) 1 μM A 23 187.

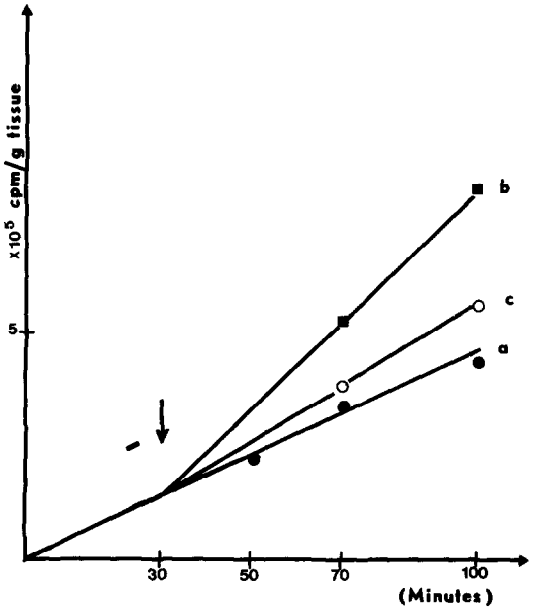

Fig. 5. Effect of carbachol and A 23 187 on [^{32}P]phosphate incorporation into total proteins. Carbachol or ionophore were added at the time indicated by the arrow. (a) Control; (b) 2 μM carbachol; (c) 1 μM A 23 187.

Hence it seems that carbachol or ionophore A 23 187, by increasing the intracellular level of calcium, could activate the phosphorylase-kinase system [14] and enhance the rate of glycogen degradation. According to these results, it cannot be decided whether glycogen biosynthesis as such is affected.

3.3. *Phospholipids*

In rat parotid gland carbachol enhances the phosphate turnover of phospholipids whereas the ionophore A 23 187 is without effect (fig. 4). Hence it appears that calcium uptake induced by the ionophore A 23 187 does not simulate the cholinergic agonists effect on the phosphate turnover of phospholipids whereas it mimics the mediators effect on protein secretion.

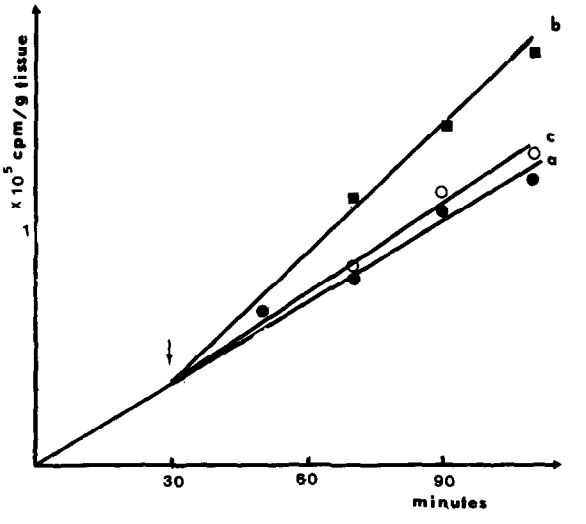

Fig. 6. Effect of carbachol and A 23 187 on [^{32}P]phosphate incorporation into 34 000 *g* supernatant proteins. Carbachol or ionophore were added at the time indicated by the arrow. (a) Control; (b) 2 μM carbachol; (c) 1 μM A 23 187.

3.4. *Protein phosphorylation*

Fig. 5 demonstrates that A 23 187 and carbachol enhance the velocity of phosphorylation of total proteins in rat parotid gland. However carbachol seems to be more potent than ionophore in this process, since it simulates the velocity by 100% whereas A 23 187 increases it only by 30%. Indeed, as shown in fig. 6, the cholinergic agonist stimulates the protein phosphorylation in the 34 000 *g* supernatant, but A 23 187 is ineffective in this process. The radioactivity cannot be due to [^{32}P]phosphate incorporated into phospholipids or into nucleic acids since TCA sediments have been treated with organic solvent and heated in TCA. The linkage between [^{32}P]phosphate and protein is acid stable; on the other hand [^{32}P] phosphate is completely eliminated by alkali treatment (results not shown). Hence it may be postulated that [^{32}P]phosphate is bound to serine or threonine residues.

We have verified that the phosphorylation of proteins and the phospholipidic effect are not due to variation of the level or phosphate turnover of nucleotides: there is no effect of carbachol or ionophore on these parameters. This latter result is in agreement with those of Hokin [5].

4. Discussion

Carbachol induces protein secretion, glycogen breakdown and stimulates phosphate turnover in phospholipids and protein phosphorylation. We demonstrated that carbachol-induced protein discharge is not reduced by adrenergic blockers (propranolol or phentolamine) but atropine inhibits it completely [4,15]. Similar results have been obtained on glycogen and phospholipid metabolism and protein phosphorylation (results to be published). These data show that the phenomena, described above, induced by carbachol are due to the direct action of the agonist at the cholinergic receptor level. In rat parotid glands, the regulation of secretory processes by cholinergic agonists seems to be in connection with a physiological function of parasympathetic nerve endings as observed by Hand [16]. In the course of cholinergic stimulation, it appears that the mediator does not evoke a variation of cAMP concentration [8] but rises the cGMP intracellular level [11] and increases the calcium uptake as G. Keryer has shown in the parotid gland [10]. According to data reported in the present paper, cGMP is inefficient in inducing protein secretion. On the other hand A 23 187 which is known to activate calcium movements through membranes [17], mimics the effect of carbachol on protein discharge and glycogen metabolism. Thus calcium could play the role of an intracellular messenger of cholinergic agonists in these processes. However A 23 187 does not mimic the carbachol effects on the phosphate turnover in phospholipids. Although in the absence of Ca^{2+} ions, this effect is greatly diminished, as shown by Hokin [7], according to these observations it could be suggested that carbachol and calcium must both the present in order to elicit the phospholipidic response. In addition carbachol is more potent than the ionophore to stimulate the velocity of total protein phosphorylation and the cholinergic agonist induces protein phosphorylation in the 34 000 *g* supernatant, whereas A 23 187 is ineffective in this process. Hence, two kinase systems regulated by parasympathetic mediators, seem to be present in parotid glands. Our results suggest that one of these systems may be directly activated by calcium alone like the phosphorylase kinase system [14]. This system could be involved in microtubule or microfilament phosphorylation during protein discharge as we proposed previously [4]. The other kinase system is not solely calcium-dependent but requires carbachol stimulation : a role of cGMP could be postulated in the process. This second kinase system could be involved in events connected with other cholinergic stimulations (for instance phospholipid turnover) but not directly with protein discharge.

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