Effects of Ca^{2+} and Calmodulin Antagonists on the Oxygen Uptake Induced by Acetylcholine or Substance P in Rat Submandibular Gland Slices

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Abstract—Effects of Ca^{2+} and calmodulin antagonists on the oxygen uptake induced by acetylcholine (ACh) or substance P (SP) were investigated in rat submandibular gland slices. The oxygen uptake induced by ACh or SP was significantly inhibited by removing Ca^{2+} from the medium and the slices. The oxygen uptake by ACh in the Ca^{2+} -deficient slices was almost completely recovered by the addition of 3.0 and 5.0 mM Ca^{2+} , whereas that by SP was not recovered by the addition of 3.0 mM Ca^{2+} , but recovered by 5.0 mM Ca^{2+} . Ca^{2+} antagonists, diltiazem, verapamil and La^{3+} , significantly inhibited the ACh-induced oxygen uptake. On the other hand, the SP-induced oxygen uptake was inhibited by diltiazem and La^{3+} , but not by verapamil. Calmodulin antagonists, trifluoperazine, chlorpromazine and W-7, had no inhibitory effects on the ACh-induced oxygen uptake. The SP-induced oxygen uptake was not affected by trifluoperazine, chlorpromazine and low concentrations of W-7, but was inhibited by high concentrations of W-7. These results suggest that the ACh- or SP-induced oxygen uptake is dependent on the presence and permeability of Ca^{2+} with a subtle difference between the ACh and the SP mechanisms and that the oxygen uptake is independent of calmodulin.

The role of the autonomic nervous system in the regulation of salivary secretion is well established. In general, mammalian salivary glands respond to the stimulation by autonomic nerves or by drugs that mimic their actions. The secretion of saliva can also be elicited, at least in some species, by certain peptides, particularly some that are isolated from the skin of non-mammalian species or from the intestine or the brain of mammalian species, such as physalaemin, eledoisin and substance P (SP) (1–5). In the previous paper, we demonstrated that SP was similar to physalaemin and eledoisin-related peptide in stimulating the oxygen uptake of rat submandibular gland slices (6). Energy is

important in the control of cellular functions. In salivary glands, a continuous supply of energy may be required for the secretion of various substances. Tanaka et al. (7) reported a high level of creatine phosphate and evidence showing the existence of mitochondrial creatine kinase in rat submandibular glands.

It is commonly believed that calcium (Ca^{2+}) is intimately involved in the coupling between stimulation and secretion in secretory organs (8). In particular, cytoplasmic free- Ca^{2+} is considered to trigger the release of amylase from salivary glands (9). However, the regulatory system controlling the cytoplasmic free- Ca^{2+} is not fully known. More recently, calmodulin has been found to mediate many of the Ca^{2+} effects in cellular functions (10, 11). It is suggested that calmodulin plays an important role in the

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release of prolactin from the pituitary gland and that of insulin, glucagon and amylase from the pancreas (12–14). In addition, the rat salivary glands have been shown to contain calmodulin (15, 16). However, it is not adequately investigated whether calmodulin is involved in the respiratory and secretory mechanisms of rat submandibular gland slices.

In the present report, we have studied the mechanism by which acetylcholine (ACh) or SP stimulates the respiration of rat submandibular gland slices, with particular emphasis on the possible roles of Ca^{2+} and calmodulin.

Materials and Methods

Male rats of the Wistar strain (weighing about 200–250 g) were used. The animals had access to a standard pelleted diet and to water ad libitum. They were sacrificed by decapitation. Immediately following the dissection, each gland was cut into slices with a Stadie-Riggs slicer (slice thickness ca. 0.5–0.8 mm) and placed in the medium. Then each slice of approximately 60–80 mg was immersed in the Warburg vessel containing 3 ml of physiological solution (Krebs-Ringer phosphate buffer, pH 7.4). The solution was continuously gassed with pure oxygen. The composition of the medium was modified in some experiments. The Ca^{2+} -free medium used had essentially the same

electrolyte composition as the Na^{+} -containing medium, except that the CaCl_2 was omitted. In the experiment of Ca^{2+} addition, the phosphate buffer in the medium was replaced with Tris-HCl buffer. The oxygen uptake was measured by Warburg's manometric method under pure oxygen for 60 min at 37.5°C. The addition of reagents was made after preincubation for 10 min.

The following agents were used in the present experiment: ACh (Daiichi Pharmaceutical Co., Ltd), SP (Protein Research Foundation, Japan), diltiazem (Tanabe Pharmaceutical Co., Ltd.), verapamil (Eizai Co., Ltd.), trifluoperazine (Yoshitomi Pharmaceutical Co., Ltd.), chlorpromazine (Shionogi Pharmaceutical Co., Ltd.), and W-7 (Rikaken Co., Ltd.). All the other chemicals used were of analytical grade.

Results are expressed as the mean $\pm$ S.E. of several experiments, and are analyzed by Student's *t*-test.

Results

Influence of Ca^{2+} removal on the oxygen uptake induced by ACh or SP: In the previous report (6), we showed that the addition of ACh or SP caused a linear increase in the oxygen uptake and that the obvious effects due to these agents were observed in the range of 10–15 μM . On the basis of this result, the present experiment was made in the presence of 10 μM ACh or 15 μM SP.

Table 1. Effects of Ca^{2+} removal on the oxygen uptake induced by ACh or SP

Conditions		Oxygen uptake ($\mu\text{lO}_2/100 \text{ mg/hr}$)
Control	Normal medium+Normal slice	141 $\pm$ 4
	Ca^{2+} -free medium+Normal slice	140 $\pm$ 3 NS
	Ca^{2+} -free medium+ Ca^{2+} -deficient slice	104 $\pm$ 2*
Acetylcholine, 10 μM	Normal medium+Normal slice	194 $\pm$ 7
	Ca^{2+} -free medium+Normal slice	142 $\pm$ 1*
	Ca^{2+} -free medium+ Ca^{2+} -deficient slice	156 $\pm$ 2*
Substance P, 15 μM	Normal medium+Normal slice	194 $\pm$ 4
	Ca^{2+} -free medium+Normal slice	145 $\pm$ 3*
	Ca^{2+} -free medium+ Ca^{2+} -deficient slice	138 $\pm$ 2*

The Ca^{2+} -free medium was prepared as described in "Materials and Methods". The Ca^{2+} -deficient slices were prepared in the medium containing 2 mM EGTA for 60 min at 4°C. Normal slices were not treated with EGTA. Each value represents the mean $\pm$ S.E. for seven experiments. NS, not significant.

* $P < 0.01$.

Removal of Ca^{2+} from the medium significantly blocked the stimulation of ACh or SP (Table 1). Furthermore, the Ca^{2+} -deficient slices were prepared by immersing them in the medium containing 2 mM EGTA for 60 min at 4°C. The oxygen uptake due to ACh or SP in the Ca^{2+} -deficient slices was similar to the result observed in the Ca^{2+} -free medium. The addition of ACh or SP to the Ca^{2+} -free medium stimulated the oxygen uptake in the Ca^{2+} -deficient slices. However, the level of oxygen uptake was considerably lower as compared with that seen with normal slices.

Effects of various concentrations of Ca^{2+} on the oxygen uptake in the Ca^{2+} -deficient slices: Figure 1 shows the effects of various concentrations of Ca^{2+} on the oxygen uptake stimulated by ACh or SP in the Ca^{2+} -deficient slices. The ACh-induced oxygen uptake was completely recovered by increasing Ca^{2+} concentration to 3.0 and 5.0 mM. On the other hand, the recovery of the SP-induced oxygen uptake was poor compared with that of ACh response and the reversibility of SP response was observed at 5.0 mM Ca^{2+} .

Effects of Ca^{2+} antagonists on the oxygen uptake induced by ACh or SP: Diltiazem, verapamil and La^{3+} have been shown to

possess Ca^{2+} -antagonistic properties in several organs (17–19). The Ca^{2+} antagonists were tested for their effects on the oxygen uptake (Table 2). The presence of any of the three Ca^{2+} antagonists in the medium significantly reduced the oxygen uptake induced by ACh, while the SP-induced oxygen uptake was strongly inhibited by

Fig. 1. Effects of various concentrations of Ca^{2+} on the oxygen uptake in the Ca^{2+} -deficient slices. The oxygen uptake induced by the agonists in the normal medium was taken as 100%, and the recovery rates are expressed as % of the respective value which was obtained by the addition of Ca^{2+} . Each point represents the mean for seven experiments.

Table 2. Effects of diltiazem, verapamil and La^{3+} on the oxygen uptake induced by ACh or SP

Conditions	Oxygen uptake ($\mu\text{lO}_2/100 \text{ mg/hr}$)
Control	146±1
+Diltiazem, 100 μM	140±5 NS
+Verapamil, 100 μM	142±2 NS
+ La^{3+} , 3 mM	149±5 NS
Acetylcholine, 10 μM	194±7
+Diltiazem, 10 μM	158±6 *
+Diltiazem, 100 μM	150±3 *
+Verapamil, 10 μM	165±2 *
+Verapamil, 100 μM	140±2 *
+ La^{3+} , 3 mM	155±2 *
Substance P, 15 μM	194±4
+Diltiazem, 10 μM	187±6 NS
+Diltiazem, 100 μM	136±4 *
+Verapamil, 10 μM	196±4 NS
+Verapamil, 100 μM	194±3 NS
+ La^{3+} , 3 mM	139±3 *

Each value represents the mean±S.E. for seven experiments. NS, not significant. * $P<0.01$.

Table 3. Effects of trifluoperazine, chlorpromazine and W-7 on the oxygen uptake induced by ACh or SP

Conditions	Oxygen uptake ($\mu\text{I}\text{O}_2/100 \text{ mg/hr}$)
Control	145 $\pm$ 4
+Trifluoperazine, 100 μM	151 $\pm$ 3 NS
+Chlorpromazine, 10 μM	144 $\pm$ 3 NS
+W-7, 8.9 μM	145 $\pm$ 3 NS
Acetylcholine, 10 μM	195 $\pm$ 3
+Trifluoperazine, 100 μM	199 $\pm$ 2 NS
+Chlorpromazine, 10 μM	188 $\pm$ 4 NS
+W-7, 8.9 μM	193 $\pm$ 5 NS
+W-7, 89 μM	197 $\pm$ 3 NS
Substance P, 15 μM	194 $\pm$ 4
+Trifluoperazine, 100 μM	181 $\pm$ 2 NS
+Chlorpromazine, 10 μM	195 $\pm$ 6 NS
+W-7, 8.9 μM	189 $\pm$ 3 NS
+W-7, 89 μM	162 $\pm$ 2 *

Each value represents the mean $\pm$ S.E. for seven experiments. NS, not significant. * $P<0.01$.

diltiazem and La^{3+} , but was not inhibited by verapamil. The inhibitory effect of diltiazem or La^{3+} was modified by increasing Ca^{2+} concentration of the medium to 2.5–8.0 mM (data not shown). These antagonists alone had no significant effect on the oxygen uptake in the absence of ACh or SP.

Effects of calmodulin antagonists on the oxygen uptake induced by ACh or SP: Phenothiazines, trifluoperazine and chlorpromazine, and W-7 have been shown to function as calmodulin antagonists (13, 14, 20). In order to determine whether calmodulin was involved in the mechanism of the increase of the oxygen uptake stimulated by ACh or SP, we studied the effects of several calmodulin antagonists (Table 3). The ACh-stimulated oxygen uptake was not inhibited by the addition of these antagonists. The SP-stimulated oxygen uptake was not inhibited by trifluoperazine, chlorpromazine and low concentrations of W-7. However, high concentrations of W-7 reduced SP response.

Discussion

The study of Terroux (21) on dog submandibular glands demonstrated that an approximately linear relationship existed between the rate of saliva flow and energy expenditure during the chorda tympani stimu-

lation. We found that oxidative phosphorylation inhibitors, oligomycin and antimycin, significantly inhibited the release of amylase and sialic acid and the oxygen uptake stimulated by pilocarpine or isoproterenol in dog submandibular gland slices (Komabayashi, T., unpublished). Therefore, the mechanism which causes the secretion should be related to the increment of the oxygen uptake. The oxygen uptake in rat submandibular gland slices is stimulated by various agents. For example, the oxygen uptake by adrenergic agonists is mediated by α - and β -receptors (22), while that due to cholinergic agonists is mediated by muscarinic receptors (6).

Some previous studies on the salivary glands indicate that Ca^{2+} plays an important role in fluid secretion. First, the secretion by α -adrenergic or cholinergic agonists is inhibited by Ca^{2+} removal (19, 23). Secondly, these agonists act by increasing the rate of Ca^{2+} transfer into the cells of the salivary glands through an increase of the cell membrane permeability for Ca^{2+} (9, 24). SP also causes the Ca^{2+} -dependent release of substances from salivary glands (25). Thus, the action of SP and that of α -adrenergic or cholinergic agonists are similar with respect to Ca^{2+} requirement. In the present study, we found that removal of Ca^{2+}

from the medium inhibited the action of ACh or SP on the oxygen uptake (Table 1). These findings differ from the result in the previous report (26) that the effects of adrenaline and noradrenaline on the oxygen uptake did not require extracellular Ca^{2+} . Basal oxygen uptake in the Ca^{2+} -deficient slices significantly decreased in the Ca^{2+} -free medium. In these conditions, the stimulant effect of ACh or SP was observed. However, the level of oxygen uptake by ACh or SP was considerably lower as compared with that seen with normal slices. This phenomenon of ACh or SP stimulating the oxygen uptake in the absence of Ca^{2+} can not be explained in this study, and further study is required. In addition, diltiazem and La^{3+} (18, 27) which are known to be specific antagonists of Ca^{2+} -flux significantly inhibited the effects of ACh or SP on the oxygen uptake (Table 2), and these inhibitory effects were modified by increasing the Ca^{2+} concentration of the medium. These results suggest that the action of ACh or SP strongly depends on the influx of external Ca^{2+} into the secretory cells.

A recent experiment indicates that there might be some subtle difference between the action of SP and that of carbachol in the requirement for Ca^{2+} . Miller et al. (28) reported that the local anesthetic tetracaine, a compound which blocks Ca^{2+} -flux, blocked the effect of carbachol on $^{45}\text{Ca}^{2+}$ -flux changes and amylase secretion, while it did not inhibit the effects of SP. In the present experiment, we also showed that a Ca^{2+} -flux antagonist, verapamil, inhibited the effects of ACh on the oxygen uptake, while it did not inhibit the effects of SP (Table 2). Furthermore, in the reversibility of each response in the Ca^{2+} -deficient slices, the reversibility of the SP response was poor compared with that of the ACh response, and then high concentrations of Ca^{2+} were required for the SP response (Fig. 1). From these results, the SP mechanism may be qualitatively different from the ACh mechanism in the transduction mechanism of Ca^{2+} .

The phenothiazines and W-7 were used as an approach to determine whether calmodulin had a role in several organs (12–14, 16, 29, 30). Kanagasuntheram and Teo

(16) showed that the activation of calmodulin in rat salivary glands was Ca^{2+} -dependent and was dose-dependently inhibited by trifluoperazine which blocked the amylase release induced by isoproterenol, dibutyryl cyclic AMP, carbachol and phenylephrine. In contrast, Spearman and Butcher (29) reported that the phenothiazines, trifluoperazine, chlorpromazine and thioridazine, failed to inhibit the amylase release induced by dibutyryl cyclic AMP, but W-7 partially inhibited the release induced by agent. They also suggested that the slight inhibition of the amylase release seen with W-7 was due to the toxic effect of this compound. Thus, there are conflicting reports. In our experiment, trifluoperazine, chlorpromazine and W-7 did not inhibit the oxygen uptake induced by ACh or SP, except that the SP response was blocked by high concentrations of W-7 (Table 3). The effect at high concentrations of W-7 is presumably due to nonspecific effects of this agent. These results are similar to those obtained by Spearman and Butcher (29) and suggest that calmodulin may not be involved in the secretory and respiratory mechanisms of rat submandibular glands.

There is a possibility that Na^{+} -pump activation is involved in the mechanism utilizing the extra energy consumption during the ACh or SP stimulation in submandibular glands. First, there are high concentrations of Na^{+} - K^{+} -ATPase present in rat submandibular glands (31). In addition, ouabain, a highly specific inhibitor of the Na^{+} -pump, blocks the salivary secretion and the oxygen uptake stimulated by cholinergic agonists in cat and rat submandibular glands (32, 33). Secondly, the Na^{+} -pump activation by carbachol is Ca^{2+} -dependent (34), and as shown in Table 1 and Fig. 1, the effect of ACh or SP on the oxygen uptake is Ca^{2+} -dependent. As described above, it is thought that Na^{+} -pump activation, as well as Ca^{2+} -flux, is profoundly concerned in the stimulation of oxygen uptake by ACh or SP.

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