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Stimulation by calcium and carbamoylcholine of the ouabain-sensitive uptake of $^{86}Rb^+$ in isolated rat pancreatic acinar cells

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The uptake of $^{86}Rb^+$ was assayed in isolated rat pancreatic acinar cells to determine the effect of calcium and carbamoylcholine on the ouabain-sensitive and ouabain-insensitive components. The presence of calcium in the medium bathing the cells during the preincubation and the main incubation periods was needed to preserve in optimum conditions the uptake of $^{86}Rb^+$, the stimulation by carbamoylcholine and the sensitivity to ouabain. In the presence of calcium, the ouabain-sensitive component of $^{86}Rb^+$ uptake was higher than the ouabain-insensitive. The ouabain-sensitive component was 3-times lower in cells incubated in a medium lacking calcium and containing 1 mM EGTA, as compared to cells incubated in the presence of calcium. Carbamoylcholine, at $5 \cdot 10^{-4}$ M, stimulated the uptake of $^{86}Rb^+$ and this effect depended on the presence of calcium in the bathing medium. Maximal stimulation by carbamoylcholine was reached at 0.2 mM calcium. The nett stimulation by carbamoylcholine was inhibited up to 85% by 1 mM ouabain. As judged by digitonin-disruption of plasma membrane, the above-indicated effects were limited to a cytoplasmic pool of $^{86}Rb^+$ and a leaky plasma membrane could be ruled out. The results suggest that in rat pancreatic acinar cells, carbamoylcholine stimulated the ouabain-sensitive uptake of $^{86}Rb^+$ and required the presence of calcium in the bathing medium.

Introduction

In pancreas tissue, calcium has been proposed as an intracellular mediator for the increase in membrane conductance induced by agonist [1,2]. The presence of cation channels that are evoked by cholecystokinin or acetylcholine has been demonstrated in the basolateral plasma membrane of pancreatic acini [3], as well as the presence of other transport protein: the electrogenic Na^+/K^+ pump sensitive to ouabain [4]. In contrast to salivary acinar cells, in exocrine pancreas, carbachol does not modify the unidirectional efflux of K^+ [5] and also the failure of carbachol to modify intracellular Na^+, K^+ and Cl^- concentrations has been shown [6]. It remains to be elucidated whether in pancreas tissue calcium and agonists modify the activity of the ouabain-sensitive pump, as described in the rat parotid gland [7] and lacrimal gland cells [8].

The purpose of the present work is to study the effect of calcium and carbamoylcholine on ouabain-sensitive and ouabain-insensitive uptake of $^{86}Rb^+$. The data suggested that in rat pancreatic acinar cells, calcium was required for the functional integrity of the Na^+/K^+ pump and for the stimulation by carbamoylcholine of $^{86}Rb^+$ uptake.

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Abbreviations EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid

Methods

Pancreatic acinar cells were isolated from Wistar rats as described earlier [9] for the guinea-pig. Two rats were killed by decapitation under ether anaesthesia for every experiment. The uptake of $^{86}Rb^+$ was assayed by measuring the radioactivity pelleted within cells as follows: acinar cells were preincubated in 10 ml of a medium consisting of 116 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1 mM $MgCl_2$, 15 mM Hepes, 14 mM glucose, 0.1 mg/ml soybean trypsin inhibitor and 1% bovine serum albumin. The pH was adjusted to 7.4. The measured osmolarity was 278 ± 5 mosmol/l. The medium was gassed with 95% O_2 and 5% CO_2. After 30 min, the cells were sedimented and resuspended in Krebs-Ringer-Hepes. For the main incubation, aliquots of 0.2 ml of the cell suspension were added to separate vessels with the same buffer and tracer amounts of $^{86}Rb^+$. The final volume was 2.3 ml and the protein concentration 2–3.5 mg/ml. Incubation was stopped by mixing 0.2 ml of the cell suspension with 1 ml Krebs-Ringer-Hepes and centrifugation in an Eppendorf microfuge. The pellet was dissolved in 0.1 ml 1 M NaOH and counted in a β-spectrometer. The extracellular space of pelleted cells was determined with [U-^{14}C]sucrose as described earlier [9] and it was calculated to be 0.18 ± 0.04 μl/mg protein. The equivalent values of $^{86}Rb^+$ were found to be insignificant and no corrections were made for the radioactivity values obtained in the pelleted cells. The secretion of amylase was determined as described earlier [9].

The disruption of plasma membrane with digitonin was achieved by transferring 0.2-ml aliquots of cells previously loaded with $^{86}Rb^+$ (see above) to test-tubes containing 1 ml 280 mM saccharose and 2 mM EGTA.

Digitonin, at a final concentration of 0.15 mg/ml, was added by mixing the medium with the digitonin previously pipetted at the inner side of the cap of the Eppendorf tubes. After 30 s, cells were pelleted at $10000 \times g$. The water content of the pellets of digitonized and untreated cells was calculated by weighing the pellets before and after drying overnight at 95°C and expressed as μl water/mg dry wt. $^{86}Rb^+$ content was expressed as the distribution ratio: cpm/μl water in the pellet/cpm per μl of the incubation medium. The disrupting effect of digitonin was assayed by measuring the release of intracellular enzymes [10].

Mg^{2+}-dependent, $(Na^+ + K^+)$-ATPase was assayed as described earlier [10]. Rat pancreas tissue was homogenized as described earlier [9] for guinea-pig pancreas. A crude particulate fraction was obtained by centrifugation of the homogenate at $1500 \times g$ to remove debris followed by centrifugation at $5000 \times g$ for 30 min.

Chemicals. EGTA, Hepes and digitonin were obtained from Sigma; collagenase from Serva; ATP from Boehringer Mannheim; [^{32}P]ATP, [U-^{14}C]-sucrose and $^{86}Rb^+$ from Amersham International.

Results

The viability of the isolated rat pancreatic acinar cells, was judged by Trypan blue exclusion. More than 90% of the cells preserved their integrity. The possible damage of the plasma membrane during incubation periods was assayed by the release of lactate dehydrogenase into the medium. The total lactate dehydrogenase content of the cells was 0.45 ± 0.05 U/mg. After 60 min incubation, less than 8% of the cellular lactate dehydrogenase was released into the medium. This amount was not modified by the effectors used in the present study. As can be seen in Fig. 1, the rate of amylase secretion was stimulated by 0.5 mM carbamoylcholine; this effect was more evident during the first minutes after the addition of the secretagogue. Vasoactive intestinal peptide at the concentration tested, $2.5 \cdot 10^{-7}$ M, was a less potent secretagogue. Ouabain elicited a small increase in amylase output over the basal secretion but did not modify the release of amylase induced by carbamoylcholine (data not shown). The enzyme secretion induced by carbamoylcholine was also assayed by measuring the rate of lipase release, these results being quite similar to those obtained for amylase.

To measure the time-course of the effect of calcium and carbamoylcholine on $^{86}Rb^+$ uptake, the cells were preincubated for 30 min in a medium devoid of calcium but containing 1 mM EGTA. After washing out the preincubation medium, aliquots of the cell suspension were incubated up to 60 min under the conditions indicated in Fig. 2.

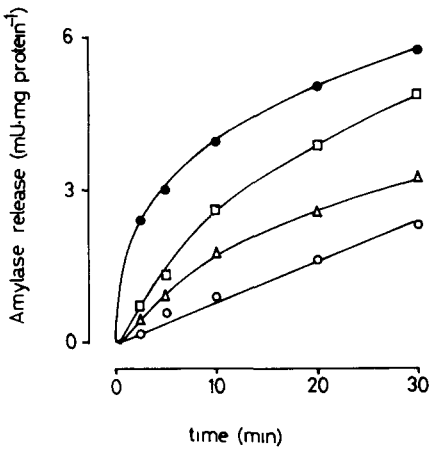

Fig 1 Amylase secretion in isolated rat pancreatic acinar cells Preincubation and incubation medium contained 1 25 mM $CaCl_2$ The incubation medium was supplemented as follows: (○) no additions, (●) $5 \cdot 10^{-4}$ M carbamoylcholine, (□) 2 5 10^{-7} M vasoactive intestinal peptide, (▲) 0 12 mM ouabain Each point represents the mean of three separate experiments Standard error was less than 11% of the mean

In the presence of 1 mM calcium, the transport rate of $^{86}Rb^+$ was increased and this effect was enhanced by carbamoylcholine. In the absence of calcium, carbamoylcholine did not modify the uptake of $^{86}Rb^+$.

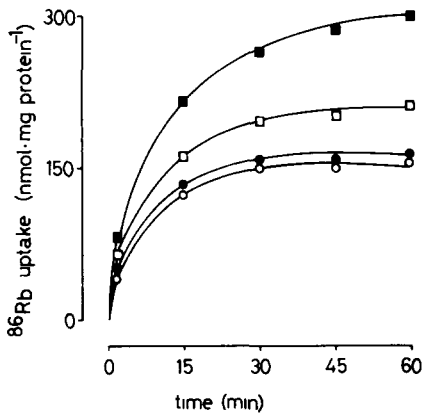

Fig. 2 Time-course of $^{86}Rb^+$ uptake. Effect of calcium and carbamoylcholine Cells were preincubated in a medium devoid of calcium and containing 1 mM EGTA The incubation medium contained 1 mM EGTA and no $CaCl_2$ (○,●), or 1 25 mM $CaCl_2$ without EGTA (□,■), in the presence (●,■) or in the absence (○,□) of $5 \cdot 10^{-4}$ M carbamoylcholine Results are the mean of four separate experiments Standard error was less than 15% of the mean

In one set of experiments, the calcium dependence of $^{86}Rb^+$ uptake was assayed following a preincubation of the cells in a medium devoid of calcium and supplemented with 1 mM EGTA. The results in Fig. 3 show the effect of calcium and carbamoylcholine on $^{86}Rb^+$ uptake. Calcium increased the uptake of $^{86}Rb^+$ over control values obtained in the presence of 1 mM EGTA. No further enhancement was observed when calcium concentration was higher than 0.4 mM. Carbamoylcholine elicited an additional increase in $^{86}Rb^+$ uptake and this effect was maximal at approx. 0.2 mM calcium. It should be noted that values in Fig. 2 were obtained by substracting in each separate experiment those values obtained after 30 min incubation in the absence of calcium and the presence of 1 mM EGTA. The average of the indicated substracted values was 201 ± 25 S.E. ($N = 7$).

The time-course of the ouabain-sensitive and ouabain-insensitive $^{86}Rb^+$ uptake in cells preincubated and incubated in the presence of 1.25 mM

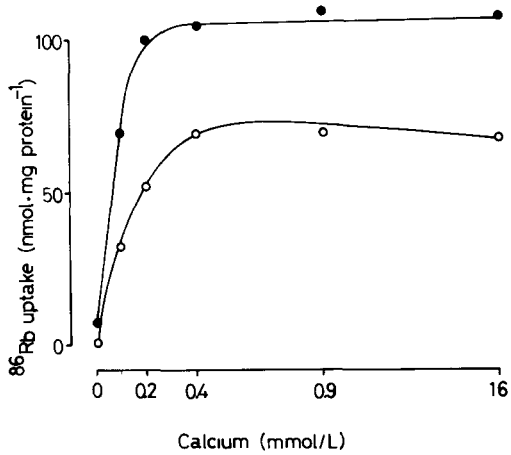

Fig 3 Calcium dependence of $^{86}Rb^+$ uptake Effect of carbamoylcholine. Cells were preincubated in a medium lacking calcium and containing 1 mM EGTA $^{86}Rb^+$ uptake was measured after 30 min incubation in the absence (○) or in the presence (●) of $5 \cdot 10^{-4}$ M carbamoylcholine and the indicated calcium concentration The values of $^{86}Rb^+$ uptake obtained in cells incubated without calcium and containing 1 mM EGTA were substracted in order to evaluate the net effect of calcium and carbamoylcholine See also text for details. Results are the mean of four experiments Standard error was less than 12% of the mean

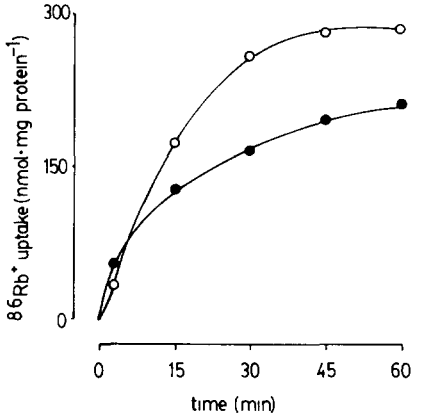

Fig 4 Time-course of ouabain-sensitive and ouabain-insensitive $^{86}Rb^+$ uptake Preincubation and incubation medium contained 1 25 mM $CaCl_2$ The uptake of $^{86}Rb^+$ was assayed in the absence or in the presence of 0 12 mM ouabain Ouabain-sensitive $^{86}Rb^+$ uptake (○) refers to values obtained in the absence of ouabain minus those obtained in the presence of ouabain Ouabain-insensitive $^{86}Rb^+$ uptake (●) refers to values obtained in the presence of ouabain Values are the mean of four separate experiments Standard error was less than 12% of the mean

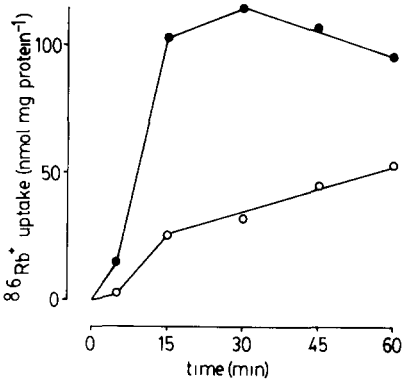

Fig 5 Inhibition by ouabain of carbamoylcholine-stimulated $^{86}Rb^+$ uptake Preincubation and incubation medium contained 1 25 mM $CaCl_2$ The net effect of 5 10^{-4} M carbamoylcholine was calculated by subtracting the values obtained without the secretagogue to those obtained with the secretagogue Ouabain was present (○) or absent (●) in control and secretagogue-stimulated cells Single experiment representative of two others

calcium was studied. As may be seen in Fig. 4, an apparent equilibrium was reached after 40 min. Although the half-time was quite similar in both components, approx. 10 min, the higher values reached in the ouabain-sensitive component can be appreciated. Under these conditions, the net effect of carbamoylcholine was a significant increase of the ouabain-sensitive $^{86}Rb^+$ uptake, that is, the stimulation by carbamoycholine was inhibited up to 75% by ouabain (see Fig. 5).

Table I summarizes the effect of calcium on ouabain-sensitive and ouabain-insensitive components of $^{86}Rb^+$ uptake. The absence of calcium reduced the uptake of $^{86}Rb^+$ by the ouabain-sensitive component to 0.34-times, whereas the ouabain-insensitive component was reduced to 0.71-times their respective maximal uptake in the presence of calcium. In other words, the degree of

TABLE I

EFFECT OF CALCIUM ON OUABAIN-SENSITIVE AND OUABAIN-INSENSITIVE $^{86}Rb^+$

Cells were preincubated for 30 min. After washing out the medium, cells were incubated for 60 min with tracer amounts of $^{86}Rb^+$. Results in A and B were obtained from the same cell preparations. The differences between ouabain-sensitive and ouabain-insensitive were analyzed by the paired Student's *t*-test ($P < 0.02$–0.05). The difference of the means A vs. B of the ouabain-insensitive component was not significant. Results are given in nmol $^{86}Rb^+$/mg protein per 60 min Mean ± S E of four experiments

	Conditions		$^{86}Rb^+$ uptake	
	preincubation	incubation	ouabain-sensitive	ouabain-insensitive
A	No $CaCl_2$ + 1 mM EGTA	No $CaCl_2$ + 1 mM EGTA	98 ± 6	152 ± 16
B	No $CaCl_2$ + 1 mM EGTA	1 25 mM $CaCl_2$ No EGTA	150 ± 11	204 ± 17
C	1.25 mM $CaCl_2$ No EGTA	1.25 mM $CaCl_2$ No EGTA	286 ± 15	214 ± 19

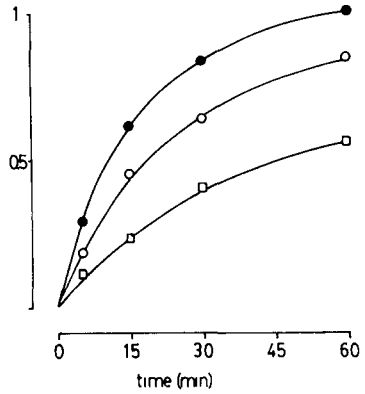

Fig. 6 Effect of calcium and carbamoylcholine on the ouabain-sensitive uptake of $^{86}Rb^+$ Cells were preincubated with 1 mM EGTA and no $CaCl_2$ The uptake of $^{86}Rb^+$ was assayed in the absence or in the presence of 0.12 mM ouabain under the following conditions. (□) no $CaCl_2$ plus 1 mM EGTA, (○) no EGTA plus 1 25 mM $CaCl_2$, (●) no EGTA plus 1 25 mM $CaCl_2$ and $5 \cdot 10^{-4}$ M carbamoylcholine Values in the ordinate axis are given as the ratio V/M V refers to values in nmol $^{86}Rb^+$/mg protein at the indicated time intervals and M refers to values obtained in the presence of calcium and carbamoylcholine at 60 min, calculated as described in Fig 4 Values of M averaged 174 ± 8 ($n = 4$)

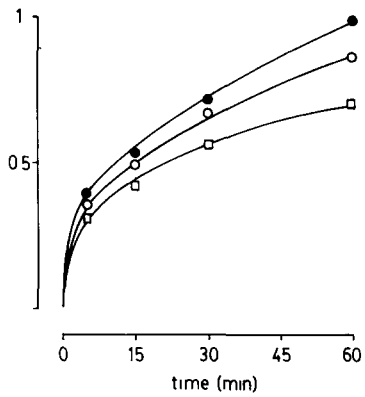

Fig 7 Effect of calcium and carbamoylcholine on the ouabain-insensitive uptake of $^{86}Rb^+$ Results refer to values obtained in the presence of 0 12 mM ouabain and other conditions as in Fig 6 Values of M averaged 236 ± 21 ($n = 4$)

inhibition by ouabain of $^{86}Rb^+$ uptake was higher when the preincubation and the incubation medium contained calcium. Experiments were designed in order to study the effect of calcium and carbamoylcholine on the ouabain-sensitive and ouabain-insensitive components of $^{86}Rb^+$ uptake. With this in mind, cells were preincubated in a medium devoid of calcium and containing 1 mM EGTA. The uptake of $^{86}Rb^+$ was assayed under the conditions incidated in the legends to Figs. 6 and 7. The most significant feature was that the relative stimulation by calcium and by carbamoyl-

TABLE II

EFFECT OF OUABAIN ON THE STIMULATION BY CALCIUM AND CARBAMOYLCHOLINE OF $^{86}Rb^+$ UPTAKE

Aliquots of the cell suspension from each preparation were preincubated in A and B in the absence of calcium and the presence of 1 mM EGTA and in C in the presence of 1.25 mM $CaCl_2$. The main incubation was carried out under the above indicated conditions. Control tubes contained during the main incubation 1 mM EGTA for A and B and 1 25 mM $CaCl_2$ for C, their corresponding values, in the absence or in the presence of ouabain, were substracted to obtain the indicated values. Results are from experiments of Figs. 5, 6 and 7 and additional experiments with 1 mM ouabain and $1 \cdot 10^{-6}$ M carbamoylcholine. Values are the mean ± S.E. of the number of experiments given in parentheses.

	Additions	nmol $^{86}Rb^+$/mg protein per 30 min		
		none	0.12 mM ouabain	1 mM ouabain [b]
A	1 25 mM $CaCl_2$	62 ± 7 (8) [a]	37 ± 6 (8)	27 ± 6 (4)
B	1.25 mM $CaCl_2$ + $5 \cdot 10^{-4}$ M carbamoylcholine	114 ± 12 (8)	42 ± 5 (8)	33 ± 7 (4)
	1 25 mM $CaCl_2$ + $1 \cdot 10^{-6}$ M carbamoylcholine	127 ± 14 (4)	46 ± 9 (4)	29 ± 6 (4)
C	5 10^{-4} M carbamoylcholine	108 ± 12 (8)	31 ± 7 (8)	15 ± 4 (4)
	$1 \cdot 10^{-6}$ M carbamoylcholine	116 ± 15 (4)	27 ± 6 (4)	16 ± 4 (4)

[a] The stimulation by carbamoylcholine was statistically significant ($P < 0.05$, unpaired t-test vs. a)

[b] $P < 0.05$, paired t-test (vs. 0.12 mM ouabain).

choline was significantly higher in the ouabain-sensitive component. Some experiments were performed with 1 mM ouabain. The results (see Table II) show a nearly total inhibition of the carbamoylcholine-stimulated uptake of $^{86}Rb^+$.

The inhibition by ouabain of the $(Na^+ + K^+)$-Mg^{2+}-ATPase was assayed in a crude particulate preparation enriched in plasma membranes by 4.5-times in relation to the homogenate. The highest inhibition, up to 85%, was reached at 0.1 mM ouabain and was not modified at higher ouabain concentrations. In this membrane fraction, carbamoylcholine failed to modify the ATPase activity.

A possible subcellular compartmentation of $^{86}Rb^+$ was checked by disrupting the plasma membrane with digitonin after loading the cells with $^{86}Rb^+$. The radioactivity pelleted in digitonin-disrupted cells was nearly 8% of that in untreated cells. Taking in account the water content of the pellets, it was possible to determine the distribution ratio of $^{86}Rb^+$ in untreated and digitonized cells. Table III shows that the disruption of plasma membrane leads to the loss of $^{86}Rb^+$ gradient between intra- and extracellular spaces, allowing us to exclude the possibility that a subcellular compartmentation of $^{86}Rb^+$ took place. It should be pointed out that neither carbamoylcholine nor calcium nor ouabain modified the distribution ratio of $^{86}Rb^+$ in digitonized cells.

TABLE III

EFFECT OF DIGITONIN TREATMENT ON $^{86}Rb^+$ DISTRIBUTION AND ENZYME RELEASE

The content of water in cell pellets and the distribution of $^{86}Rb^+$ was determined as described in Methods. Enzymatic activities refer to percent of total cellular content released into the medium

	Control	Digitonin-disrupted
mg protein/ mg dry wt	0.7 ±0 06	0.63±0 09
μl water/ mg dry wt	3.06±0.04	11 70±0.5
$^{86}Rb^+$ (distribution ratio)	9.7 ±1.1	1 7 ±0 5
Lactate dehydrogenase	5.2 ±0.03	98 ±2 3
Amylase	3.14±0.3	93 ±2.18
Glutamate dehydrogenase	–	7.1 ±0.2

Discussion

Rat pancreatic acinar cells, isolated as described for guinea-pig pancreas [9] maintain their functional integrity as judged by: (a) the exclusion of Trypan blue; (b) the response to secretagogues eliciting an increase in amylase release; (c) the ability to retain the $^{86}Rb^+$ taken up from the incubation medium. $^{86}Rb^+$ has been shown to serve as a good tracer in monitoring the movements of K^+ through the plasma membrane of acinar cells [11].

The amylase secretion induced by carbamoylcholine was inhibited by atropine, but not that induced by ouabain (data not shown). The effect of ouabain is not easy to explain unless an increase in intracellular Na^+ could lead to a release of calcium from intracellular stores [12,13]. In guinea-pig pancreatic acinar cells (unpublished data, see also Ref. 9), ouabain failed to stimulate amylase secretion and the release of $^{45}Ca^{2+}$. In rat pancreas acinar cells, the sensitivity to Na^+ of mitochondrial or other subcellular stores of calcium remains to be established, although this possibility has been pointed out [13].

The amount of $^{86}Rb^+$ taken up by cells was quite variable with different cell batches. When several experiments were pooled, the variations between these experiments masked the differences seen with the effectors in an individual experiment. Therefore, results on $^{86}Rb^+$ uptake are presented in some instances as the net effect of effectors or as values relative to maximum obtained in a given experiment. The results in Fig. 5, clearly show a sensitivity to ouabain of the net stimulation by carbamoylcholine of $^{86}Rb^+$ uptake. Because these cells were incubated and preincubated in calcium-containing medium, and it has been shown that calcium increases $^{86}Rb^+$ permeability in acinar cells [7], experiments were designed in order to delimit the partial contribution of calcium and carbamoylcholine to $^{86}Rb^+$ uptake. These protocols needed the preincubation of the cells in a medium lacking calcium and containing EGTA. Several reports [9,14–16] have shown that the incubation of acinar cells under these conditions,

does not affect the functional integrity, but recently it has been shown [17] that washing out of cells in calcium-free solutions leads to a leakiness of cells which was not reversible upon readdition of calcium. The data reported by Streb and Schulz [17] contrast with the results in the present paper. A leakiness of plasma membrane would lead to the dissipation of $^{86}Rb^+$ (potassium) gradient and the cells would be unable to take up $^{86}Rb^+$. This was the case in digitonin-disrupted cells with a $^{86}Rb^+$ distribution ratio nearly 1 and the release of nearly 100% of total content of lactate dehydrogenase and amylase, in contrast with untreated cells (see Table III). These experiments excluded the possible leakiness of plasma membrane in untreated cells and a possible subcellular compartmentation of $^{86}Rb^+$. Whether the lack of glucose in the medium (see Ref. 17) could explain the differences with our data on the integrity of plasma membrane, remain to be tested. These data together with those in Fig. 4 showing that in cells incubated with calcium the ouabain-sensitive component was higher than the ouabain-insensitive component and from the time dependence of $^{86}Rb^+$ uptake (Figs. 2, 6, 7) it can be concluded that it is the uptake that is decreased, rather than a less possible nonspecific leakiness of plasma membrane following calcium deprivation.

The results in the present paper show a stimulation by calcium and carbamoylcholine of the ouabain-sensitive uptake of $^{86}Rb^+$, in agreement with the data reported by Putney and Parod [7] in parotid tissue and in lacrimal gland cells [8]. In pancreas tissue [1,2] and in plasma membrane vesicles from pancreas [18], a stimulation by calcium of the permeability to Na^+ has been described. Moreover, in the basolateral plasma membrane of pancreatic acini, there are two transport proteins that have been directly demonstrated: the Ca^{2+}-activated nonselective cation channel [3] and the ouabain-sensitive Na^+/K^+ pump [4]. Stimulation increases intracellular calcium [19] and this switches on the channel with resulting passive Na^+ uptake and K^+ release. The Na^+ uptake stimulates the Na^+/K^+ pump, restoring the balance by active Na^+ extrusion and K^+ ($^{86}Rb^+$) uptake. In this way, the measurement of total intracellular K^+ [6] should not detect an increase in K^+ uptake. Therefore, we would indeed, as shown in this paper, expect a stimulant-evoked, Ca^{2+}-sensitive and ouabain-sensitive $^{86}Rb^+$ uptake.

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