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CATIONIC ENVIRONMENT AND SECRETIN SECRETION IN CANINE DUODENAL MUCOSA *IN VITRO**

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Abstract—We examined the effects of the divalent cation calcium (Ca^{2+}) and the monovalent cations potassium (K^+) and sodium (Na^+) and different modalities that affect the fluxes of these cations on immunoreactive secretin (IRS) secretion from canine duodenal mucosa *in vitro*. In the absence of extracellular Ca^{2+}, the basal IRS secretion was inhibited by 29%. Increased uptake of Ca^{2+} by passive diffusion and facilitated uptake of Ca^{2+} by ionophore A23187 had no effect on basal IRS secretion. However, Ca^{2+} channel blocking agents, $LaCl_3$ and verapamil, inhibited basal IRS secretion by 18 and 33% respectively. High concentrations of extracellular K^+ caused a dose-dependent but delayed stimulation of IRS secretion. K^+-stimulated IRS secretion was not neurally mediated. Similarly, 9-aminoacridine, which blocks K^+ exchange, mimicked the effects of K^+. Valinomycin (10^{-6} M) inhibited both K^+-stimulated and 9-aminoacridine-stimulated IRS secretion. In the absence of extracellular Na^+, there was a delayed inhibition of both basal and K^+-stimulated IRS secretion. These results suggest that changes in cationic environment are associated with alterations in the secretion of IRS. High extracellular K^+ concentration is conducive, and the absence of extracellular Na^+ and Ca^{2+} is inhibitory to IRS secretion.

Recent evidence indicates that changes in the cationic environment are associated with alterations in the secretory process of polypeptide hormones from various endocrine cells. Using rat antral mucosal preparations, Harty *et al.* [1] showed that gastrin secretion stimulated by dibutyryl cyclic adenosine monophosphate (DBcAMP) is Ca^{2+} dependent. Fiddian-Green *et al.* [2] confirmed Harty's findings that Ca^{2+} is required during stimulus–secretion coupling of gastrin. Pittenger *et al.* [13] showed a pH-dependent dual effect of Ca^{2+} on somatostatin secretion by antral mucosa. In a recent study, we showed that DBcAMP-stimulated immunoreactive secretin (IRS) secretion was not Ca^{2+} dependent [4]. However, the effects of monovalent cations on the secretion of IRS have never been examined in detail. An apparent reason for this is the lack of a suitable technique to study IRS secretion *in vitro*. Recently, we developed an *in vitro* organ culture technique [4, 5] that permits the direct assessment of IRS secretion in canine duodenal mucosa when the cationic environment of the external medium is changed.

Using this model, we examined the roles of Ca^{2+}, K^+ and Na^+ in the basal secretion of IRS by canine duodenal mucosa. First, we studied the effects of the presence or absence of Ca^{2+}, K^+ and Na^+ on basal IRS secretion. Second, we examined the effects of graded concentrations of Ca^{2+} and K^+ on IRS secretion. Third, we studied the effects of Ca^{2+} ionophore A23187, verapamil and lanthanum chloride ($LaCl_3$) in Ca^{2+}-related studies. Finally, we studied the effects of 9-aminoacridine, valinomycin and ouabain in K^+-related studies. We examined the effects of both 9-aminoacridine and valinomycin because the former agent specifically blocks K^+ permeability, thus preventing release of intracellular K^+, whereas the latter, valinomycin, increases K^+ release.

MATERIALS AND METHODS

Calcium chloride, potassium chloride, choline chloride, choline bicarbonate, ouabain, 9-aminoacridine and verapamil were obtained from the Sigma Chemical Co. (St. Louis, MO). Ionophore A23187 was obtained from Calbiochem (LaJolla, CA). Sterile 35 × 10 mm plastic culture dishes, NCTC-135, heat-inactivated fetal calf serum and a penicillin–streptomycin mixture were purchased from the Grand Island Biological Co. (Grand Island, NY).

In the Ca^{2+} studies, duodenal mucosal explants were incubated in Trowell's T8 medium [6] supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 10% heat-inactivated fetal calf serum, 10% NCTC-135, 100 units/ml of penicillin and 100 μg/ml of streptomycin. Ca^{2+} concentrations were adjusted before incubation. Control explants were incubated in the presence of 1 mM Ca^{2+}.

In K^+ studies, the duodenal mucosal explants were incubated in modified HEPES-Ringer bicarbonate buffer (HRB) containing 100 mM NaCl, 6.0 mM KCl, 1 mM $CaCl_2$, 1.13 mM $MgCl_2$, 1 mM NaH_2PO_4, 14 mM glucose, 1 mM glutamate, 25 mM $NaHCO_3$, 1 ml/100 ml essential amino acids (50×),

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1 ml/100 ml nonessential amino acids (100×), 100 units/ml penicillin, 100 μg/ml streptomycin and 0.2% bovine serum albumin (BSA). The final pH of the HRB was adjusted to 7.0, and the osmolality was approximately 300 mOsmol. When the K^+ concentration was raised, an equivalent amount of Na^+ was removed to maintain the osmolality. When the Na^+ concentration was reduced to zero, Na^+ was replaced with choline chloride and choline bicarbonate. The residual amount of Na^+ in the Na^+-free medium was 1–3 mEq/liter. All K^+-related studies were done in the presence of 1 mM Ca^{2+}.

Mucosal preparations and organ culture techniques. Duodenal mucosal explants were prepared for organ culture from female mongrel dogs (N = 15) according to methods described previously [4, 5]. All mucosal explants were taken from the midportion of the duodenum where the IRS concentrations were the highest and least variable [4]. In brief, duodenal mucosa was dissected from the muscularis and cut into several explants that measured 4 to 6 mm^3 and weighed approximately 100 mg. These were transferred immediately to petri dishes containing the culture medium. The explants were preincubated for 30 min after which fresh medium containing test agents was added. The explants were incubated for 2 hr at 37° in a humidified atmosphere in the presence of 95% O_2 + 5% CO_2.

IRS concentrations were measured in the medium following 2 hr of incubation using radioimmunoassay procedures established in our laboratory [4]. During experiments in which time–course effects were studied, the explants were incubated for 30, 60 and 120 min. IRS concentrations in the medium are expressed as picograms of IRS secreted per milligram of tissue.

Viability was estimated using trypan blue exclusion techniques [7] and by measuring lactate dehydrogenase (LDH) [8] levels in the medium.

Experimental design. The influence of Ca^{2+} on basal IRS secretion was studied by adding Ca^{2+}, in concentrations of 0, 1, 2, 10, 20, 40 and 80 mM, to the organ culture medium. The presence of a 1 mM concentration of Ca^{2+} was considered to be normal extracellular Ca^{2+} concentration. The effects of 2.5, 5 and 10 μM ionophore A23187 were studied in the presence and absence of 1 mM Ca^{2+}. The effects of 5, 10, 20 and 50 mM verapamil and of 0.1, 1.0 and 5 mM $LaCl_3$ were studied in medium devoid of phosphate.

Stock $LaCl_3$ was prepared in 45% trichloroacetic acid and A23187 in 1:100 dilution of dimethyl sulfoxide in absolute ethanol.

The influence of monovalent cations on IRS secretion was studied by incubating the explants successfully in HRB containing doubling concentrations of K^+ (0, 6, 12, 24 and 48 mM) in the presence and absence of 10^{-7} M tetrodotoxin. This concentration of tetrodotoxin was shown previously by us to inhibit gamma-aminobutyric acid-stimulated secretin secretion in dogs [5]. The effect of 9-aminoacridine (0.05, 0.1, 0.2 and 1 mM) was studied on basal IRS secretion. The effect of the K^+-ionophore valinomycin (10^{-8}, 10^{-7} and 10^{-6} M) was studied basally, following all doses of K^+-stimulated IRS secretion, and on 9-aminoacridine-stimulated IRS secretion. Additional studies were done to determine the effect of the absence of Na^+ in the incubation medium on basal and K^+-stimulated IRS secretion. Finally, experiments were done to determine the effect of graded concentrations of ouabain (10^{-7}, 10^{-6} and 10^{-5} M) on basal and K^+-stimulated IRS secretion.

All analyses were done in duplicate and all experiments were done in triplicate in each dog; at least four dogs were used for each experimental condition. Parallel control studies were done without the addition of the test agent or without the modification of the control medium in each dog and for each variable tested. The mean of three experiments in each dog was used for subsequent data analysis, with "N" equal to the number of dogs used. An analysis of variance followed by the multiple Dunnett's test [9] was used to determine how the control group differed significantly from the experimental group, and Student's Newman–Kuel's test [10] was used to determine the pairwise differences in all of the experiments.

RESULTS

Calcium-related studies. The IRS secretion in the presence of 1 mM Ca^{2+} following 2 hr of incubation was 41.5 ± 5.2 pg/mg. When explants were incubated in the absence of 1 mM Ca^{2+}, the IRS concentration in the medium was significantly lower at the end of 2 hr of incubation (29.5 ± 3.7 pg/mg, $P < 0.05$). The mean rate of IRS secretion in the control studies in the presence of 1 mM Ca^{2+} over 2 hr was 16 pg/mg/hr. In the absence of 1 mM Ca^{2+}, however, the mean rate of IRS secretion during the first hour of incubation was similar to control studies (17 ± 5 pg/mg/hr) but, during the second hour, it dropped to 9 ± 6 pg/mg/hr. Increasing the Ca^{2+} concentration in the medium up to 80 mM did not cause significant changes in IRS concentration (control 39.5 ± 6.7; with 2–80 mM Ca^{2+}, range = 43.7 to 47.6 pg/mg; N = 4 dogs).

The addition of graded concentrations of Ca^{2+} and Ca^{2+} ionophore A23187, in the presence of 1 mM Ca^{2+}, produced no change in the concentration of secretin in the medium (control = 42.6 ± 2.3; ionophore, range = 38.7 to 42.7 pg/mg; N = 4 dogs). On the other hand, in the absence of 1 mM Ca^{2+}, 10 μM ionophore A23187 caused a significant increase in IRS secretion (control with 1 mM Ca^{2+} = 39.5 ± 6.7; control without Ca^{2+} = 29.2 ± 1.8; and A23187 without Ca^{2+} = 53.1 ± 2.8 pg/mg). However, these increases in the medium were due to leakage of secretin since the concentration of LDH increased correspondingly in the medium by 33% (control without Ca^{2+} = 36 ± 10 pg/ml, A23187 without Ca^{2+} = 48 ± 13 pg/mg). In addition, the viability of the cells decreased from 95 ± 3 to 73 ± 6%.

After 2 hr of incubation, the addition of verapamil and $LaCl_3$ at concentrations of 20 μM and 5 mM decreased IRS secretion significantly ($P < 0.05$) by 18.5 and 33% respectively (control = 45.03 ± 1.7; 20 μM verapamil = 36.7 ± 2.2; 5 mM $LaCl_3$ = 30 ± 2.2 pg/mg; N = 4 dogs).

Potassium-related studies. The basal IRS secretion in the presence of 6 mM K^+ was 31 ± 3 pg/mg (N =

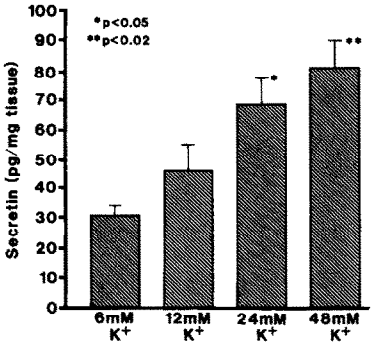

Fig. 1. Effect of graded concentrations of K^+ on secretin secretion following 2 hr of incubation. Each bar represents the mean ± SEM of eighteen explants obtained from six dogs.

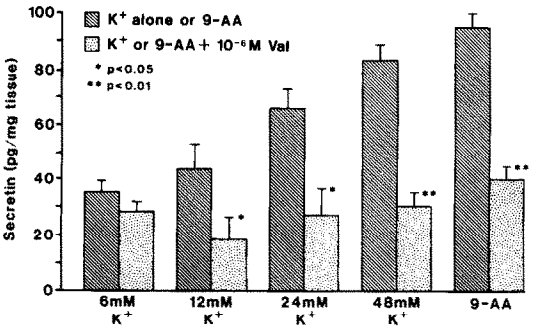

Fig. 3. Effect of 10^{-6} M valinomycin on secretin secretion stimulated by K^+ and 9-aminoacridine (9-AA). Each bar represents the mean ± SEM of twelve explants (N = 4 dogs).

6 dogs). The addition of graded concentrations of K^+ caused a concentration-dependent increase in IRS secretion (Fig. 1). Maximum secretion occurred with 48 mM K^+ (80 ± 11 pg/mg). This was a 160% increase above control levels.

Time–course effects of different concentrations of K^+ on IRS secretion are shown in Fig. 2. The addition of 12 mM K^+ did not cause a significant change in the concentration of secretin in the medium. The mean rates of secretion of IRS for both 6 and 12 mM K^+ were the same (15 ± 3.2 pg/mg/hr). The addition of 24 mM K^+ increased the IRS secretion by 100% at the end of 2 hr of incubation (control = 26 ± 5.2; 24 mM K^+ = 58 ± 11 pg/mg), whereas 48 mM K^+ increased IRS by 4-fold after 1 hr and by 3.5-fold after 2 hr compared to control levels. Exclusion of K^+ from the medium had no significant effect on the total amount or the magnitude of secretin secretion.

IRS concentrations in the presence of 10^{-7} M tetrodotoxin and 12, 24 and 48 mM K^+ were 36.9 ± 3.1, 58.51 ± 11.2 and 109.3 ± 16.8 pg/mg respectively. These values are not significantly different from control studies done with 12, 24 and 48 mM K^+ (39.7 ± 3.9, 62.9 ± 9.2 and 117.5 ± 18.1 pg/mg respectively).

The addition of 0.1 mM 9-aminoacridine stimulated IRS secretion from 35 ± 5 to 95 ± 8 pg/ml. Higher concentrations of 9-aminoacridine slightly reduced the extent of stimulation. One millimolar 9-aminoacridine had no effect on IRS secretion.

The addition of valinomycin in graded concentrations had no effect on basal IRS secretion, whereas valinomycin inhibited both K^+-stimulated and 9-aminoacridine-stimulated IRS secretion (Fig. 3). The most effective concentration of valinomycin was 10^{-6} M, which inhibited 12, 24 and 48 mM K^+-stimulated IRS secretion by 60, 56 and 52% respectively. Valinomycin at 10^{-6} M also inhibited 9-aminoacridine-stimulated IRS secretion by 58%.

Exclusion of Na^+ from the medium caused inhibition in both basal and K^+-stimulated IRS secretion (Fig. 4) gradually. The inhibition was maximal at the end of 2 hr of incubation.

The addition of ouabain in graded concentrations had no effect on basal or K^+-stimulated IRS secretion.

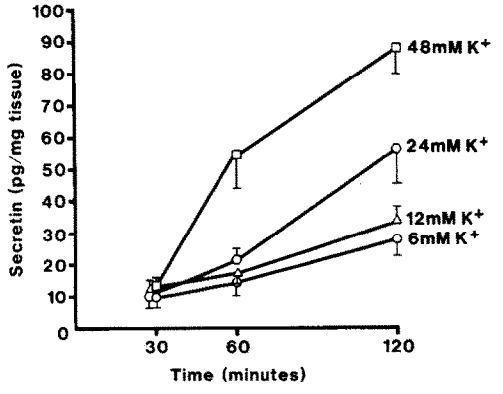

Fig. 2. Time–course of K^+-stimulated secretin secretion in canine duodenal mucosa incubated at 37° (95% O_2 + 5% CO_2) for up to 2 hr. Graded concentrations of K^+ were added at time zero. Individual sets of explants (twelve explants from four dogs per each time course) were incubated for each time point of 30 min, 1 hr and 2 hr. Each point represents mean ± SEM.

DISCUSSION

These studies demonstrated that, in the absence of Ca^{2+}, there was delayed inhibition of basal IRS

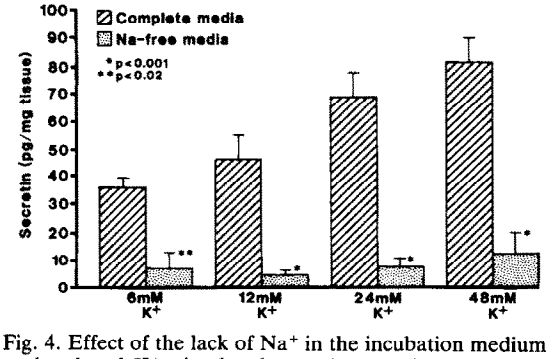

Fig. 4. Effect of the lack of Na^+ in the incubation medium on basal and K^+-stimulated secretin secretion. Each bar represents the mean ± SEM of twelve explants (N = 4 dogs).

secretion in canine duodenal mucosa *in vitro*. Increasing extracellular Ca^{2+} to pharmacologic concentrations in the medium had no significant effect on basal IRS secretion. Similarly, incubation of explants in the presence of Ca^{2+} ionophore A23187 had no significant effect. However, Ca^{2+} channel blocking agents such as verapamil and $LaCl_3$ caused delayed inhibition of basal IRS secretion.

The addition of graded concentrations of K^+ and 9-aminoacridine caused delayed increases of IRS levels in the medium. This was inhibited by the K^+-ionophore valinomycin. Exclusion of Na^+ from the medium caused delayed inhibition of both basal and K^+-stimulated IRS levels. These results suggested that alterations in cationic environment in the medium have definitive effects on IRS secretion.

Compelling evidence indicates that an increase in intracellular Ca^{2+} is an important event in the stimulus–secretion coupling of various hormones. For example, the secretion of gastrin is triggered when the intracellular concentration of Ca^{2+} is increased [1, 2]. With the exception of one study [11], which showed that intraduodenal infusion of Ca^{3+} had no effect on plasma secretin secretion, the role of Ca^{2+} in the release of IRS has never been studied.

Our study shows that both passive diffusion secondary to an excess of Ca^{2+} in the medium and facilitated uptake of Ca^{2+} induced by Ca^{2+} ionophore A23187 had no effect on IRS secretion. These findings could be interpreted to mean that there is a critical concentration of intracellular Ca^{2+} beyond which the basal secretion of IRS is not affected, However, in the absence of extracellular Ca^{2+} and in the presence of Ca^{2+} channel blocking agents, the secretion of IRS was diminished. This inhibition was not immediate but required 2 hr to be expressed. These observations could be interpreted to mean that, as the intracellular sources of Ca^{2+} are depleted, the secretin cells depend on extracellular Ca^{2+}. This suggests further that diminished intracellular Ca^{2+} inhibits basal secretion of IRS. This interpretation is consistent with our previous observations in which the BDcAMP-stimulated IRS secretion was inhibited in a delayed fashion in the absence of extracellular Ca^{2+} and IRS secretion was unchanged in the presence of an excess of Ca^{2+} [4]. Together with our previous study [4], these findings strengthen the possibility that intracellular Ca^{2+} is important for IRS secretion. However, rigorous proof will require direct measurement of intracellular Ca^{2+} and/or release studies of Ca^{2+} in isolated S cells to support this hypothesis. Presently, no such isolated cell preparation is available.

The addition of K^+ to the incubation medium in concentrations above 12 mM stimulated IRS secretion. This result is similar to observations made with insulin, glucagon and pancreatic somatostatin [12, 13].

Time–course studies done in the presence of graded doses of K^+ demonstrated that the stimulation of IRS secretion was not spontaneous. This may mean that the stimulation induced by high concentrations of K^+ in the medium was not due to depolarization. This possibility is consistent with the observation that a pharmacologic concentration of Ca^{2+} does not stimulate IRS secretion. It is possible that secretin cells in the canine duodenal mucosa do not depolarize as readily as endocrine cells in the pancreas. Proof of the membrane depolarization requires the measurement of electrochemical changes and the release of putative neurotransmitters. This is very difficult to do in a dispersed endocrine cell system such as the gastrointestinal endocrine system. It is unlikely that changes caused by high K^+ concentrations were mediated by nerves, because tetrodotoxin did not influence the secretion of IRS.

9-Aminoacridine is a planar molecule that has physical and chemical properties compatible with K^+ channels [14, 15]. This compound has been shown to cause specific blockade of K^+ channels [16], thus preventing the release of K^+. This, in turn, leads to elevation of intracellular K^+ levels which has been shown to stimulate insulin release [17]. In our study, 0.1 mM 9-aminoacridine mimicked the stimulatory effect of high K^+ on IRS secretion. This finding is similar to that observed in insulin secretion [13, 17]. Conversely, valinomycin, a K^+-specific ionophore [18] that increases the influx of K^+, inhibited both K^+- and 9-aminoacridine-stimulated IRS secretion. These findings are in agreement with studies done on pancreatic secretion of insulin and somatostatin [13]. Thus, as in insulin and somatostatin secreting cells, reduction in permeability to K^+ stimulates, while increasing permeability to K^+ inhibits, IRS secretion.

It is not possible to determine from our study the actual mechanism(s) by which K^+ stimulates IRS secretion. The mechanisms may be multifactorial and interrelated. In the islet cells, a rise in external K^+ causes increased uptake of Ca^{2+} [19]. However, our studies demonstrate that an increased influx of Ca^{2+} was not a necessary factor for stimulating IRS secretion. Therefore, it appears that the action of K^+ is independent of Ca^{2+} uptake. Other mechanisms such as alterations in the intracellular concentrations of monovalent cations, redistribution of cations in the secretin cell, and enhanced accumulation of cAMP by a Ca^{2+}-independent process [20] may have contributed to the stimulated IRS secretion. These factors need to be examined further.

Increasing extracellular K^+ is known to decrease the intracellular concentration of Na^+ and increase K^+ concentration through the activation of (Na^+, K^+)-ATPase [21, 22]. In our study, exclusion of sodium from the medium, in the presence of Ca^{2+}, inhibited both basal and K^+-stimulated secretin secretion. This effect required 2 hr to complete. On the other hand, treating explants with ouabain did not affect IRS secretion. This suggests that in the absence of Na^+, as the intracellular concentrations of Na^+ were depleted, both basal and K^+-stimulated IRS secretion required extracellular Na^+. The delayed effect caused by Na^+ suggests that it may be the intracellular Na^+, not the extracellular Na^+, that is required for IRS secretion. However, we have not directly measured sodium fluxes in our preparations to confirm this hypothesis.

In conclusion, our studies suggest that, as in other hormones, cations such as Ca^{2+}, Na^+ and K^+ may be important in the control of IRS secretion from

the S cells of canine duodenal mucosa. A lack of Ca^{2+} and Na^{+} in the medium was inhibitory, and an excess of K^{+} was stimulatory, to IRS secretion.

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