THE ROLE OF CALCIUM (Ca^{++}) IN TSH AND DIBUTYRYL 3'5' CYCLIC AMP STIMULATION OF THYROID GLUCOSE OXIDATION AND PHOSPHOLIPID SYNTHESIS

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Received October 15, 1968

The importance of calcium for the expression of hormonal effects in tissues has been repeatedly demonstrated. Omission of Ca^{++} from the buffer abolished effects of ACTH on the adrenal (Birmingham et al., 1953, and Peron et al., 1958), vasopressin on the toad bladder (Rasmussen et al., 1964) and epinephrine on amylase secretion by rat parotid gland (Rasmussen et al., 1968). In addition Ca^{++} was essential for glucose-stimulated insulin release from the pancreas (Grodsky et al., 1966) and the release of LH (Samli et al., 1968) and TSH (Vale et al., 1967) from the pituitary induced by their respective releasing factors. Bakke et al. (1957) reported that omission of Ca^{++} from the buffer decreased the weight of incubated thyroid slices and the slope of the response of this parameter to TSH. Kondo et al. (1963) noted that Ca^{++} was required for the in vitro TSH stimulation of ^{131}I transfer from the intrathyroidal iodide pool into thyroglobulin. In the absence of Ca^{++}, glucose-1-^{14}C oxidation by sheep thyroid slices was reduced as was the stimulation produced by 128 mu/ml TSH (Dumont, 1965). Current evidence indicates that since some of these hormonal effects are mediated through 3'5' cyclic AMP (Sutherland et al., 1965), Ca^{++} could be necessary for either 3'5' cyclic AMP generation or for the subsequent meta-

Supported by USPHS Grant AM-06865 from the National Institutes of Health.

bolic effects of this nucleotide. The observation of Rasmussen et al. (1968) that, in the absence of Ca^{++}, epinephrine still increased 3'5' cyclic AMP in rat parotid even though amylase secretion was inhibited suggested that Ca^{++} was essential for 3'5' cyclic AMP action. Since effects of TSH on the thyroid also appear to reflect increased 3'5' cyclic AMP production (Gilman et al., 1966), the present studies were done to evaluate the consequence of Ca^{++} exclusion on thyroid-stimulating hormone (TSH) and dibutyryl 3'5' cyclic AMP (DBC) stimulation of glucose-1-^{14}C oxidation and ^{32}P incorporation into phospholipids by dog thyroid slices.

MATERIALS AND METHODS

Dog thyroid slices were obtained and prepared as previously reported (Field et al., 1960). In order to insure that slices were being incubated in the absence of Ca^{++} in the medium, they were initially incubated for two hours in Ca^{++}-free Krebs-Ringer bicarbonate buffer containing glucose (1 mg/ml) prior to the final incubation in the same buffer during which glucose oxidation or ^{32}P incorporation into phospholipid was measured. In the initial two hour period, the buffer was changed at the end of the first and second hours. The importance of this initial incubation was emphasized by preliminary experiments in which only a single incubation in either Krebs-Ringer bicarbonate buffer containing 2.5 mM Ca^{++} (KRB) or in the same buffer minus Ca^{++} was utilized. Under such conditions frequently no difference in either baseline or TSH-stimulated glucose oxidation was observed. In order to assess further the role of Ca^{++} some slices which had been incubated initially in buffer minus Ca^{++} were transferred to buffer containing Ca^{++} for the final incubation during which glucose oxidation and ^{32}P incorporation into phospholipid was measured. When glucose oxidation was evaluated, the final incubation was for 45 minutes and 0.2 μc (200,000 cpm) glucose-1-^{14}C was added to all flasks and TSH and DBC to the appropriate flasks. The reactions were terminated and $^{14}CO_2$ collected and counted as previously described (Field et al., 1960). ^{32}P incorporation into phospholipid was

TABLE I

EFFECT OF ABSENCE OF Ca^{++} ON TSH

STIMULATION OF GLUCOSE-1-^{14}C OXIDATION IN DOG THYROID SLICES

Initial Incubation Buffer	Final Incubation Buffer	$^{14}CO_2$ Produced cpm/gm/45 min.					
		Control	TSH mu/ml				
			0.5	% Stim.	5	% Stim.	50	% Stim.
KRB	KRB	11,537 ± 1154	26,436 ± 494	130	36,565 ± 1771	220	48,536 ± 891	321
Ca^{++}-free KRB	Ca^{++}-free KRB	7,658 ± 601*	11,064 ± 703	45	17,155 ± 856	126	35,157 ± 1138	360

Each value is the average ± SEM of triplicate determinations. Slices from a single dog thyroid were used for the entire experiment.

* $p < 0.05$ comparing basal glucose oxidation in KRB with that in Ca^{++}-free KRB.

studied during a final two hour incubation (Oka et al., 1966). Each flask contained 10 μc $^{32}PO_4$ and 1 mg/ml glucose. The amounts of TSH and DBC present in the appropriate flasks are given in each table. Glucose-1-^{14}C (sp. act. 2-4 mc/mmole) was purchased from Nuclear-Chicago Corporation and ^{32}P (45 mc/mg) from Squibb. D3C was obtained from Boehringer Mannheim Corporation. TSH (2 units/mg) was a generous gift from the Endocrinology Study Section, National Institutes of Health.

RESULTS AND DISCUSSION

The data in Table I indicate that basal glucose oxidation was significantly reduced (34%) when Ca^{++} was excluded from the buffer. Furthermore, the per cent stimulation induced by 0.5 and 5 mu/ml TSH was reduced in the absence of Ca^{++}. However, a larger dose of TSH (50 mu/ml) caused an equivalent per cent increase in glucose-1-^{14}C oxidation whether Ca^{++} was present in the buffer or not. The decreased effectiveness of small amounts of TSH in the absence of Ca^{++} is not contrary to our previously published results that TSH increased glucose oxidation in a variety of buffers, many of which were devoid of Ca^{++} (O'Malley and Field, 1964). In those experiments, the TSH concentration was 250 mu/ml and the present experiments demonstrate that such large amounts of TSH were equally effective in Krebs-Ringer bicarbonate buffer with or without Ca^{++}. Although Dumont (1965) found that omission of Ca^{++} from the buffer inhibited the effect of 128 mu/ml TSH, he used sheep thyroid slices which are less sensitive to TSH than dog thyroid slices. The observation that small but not large amounts of TSH require Ca^{++} for stimulation of glucose oxidation suggests the possibility that two different mechanisms may be involved. A somewhat analagous situation has been reported with adipose tissue (Ho et al., 1967). The lipolytic effect of low doses of ACTH and catecholamines were inhibited by omission of K^+ from the buffer. However, such inhibition could be overcome by increasing the concentrations of the hormones.

Since the current concept of TSH action involves mediation by 3'5' cyclic

TABLE II

EFFECT OF ABSENCE OF Ca^{++} ON TSH AND DBC

STIMULATION OF GLUCOSE-1-^{14}C OXIDATION IN DOG THYROID SLICES

Initial Incubation Buffer	Final Incubation Buffer	$^{14}CO_2$ Produced cpm/gm/45 min.				
		Control	TSH 1 mu/ml		DBC 500 μg/ml	
				% Stim.		% Stim.
KRB	KRB	11,167 ± 554	21,800 ± 2800	95	17,800 ± 1665	60
Ca^{++}-free KRB	Ca^{++}-free KRB	7,500 ± 100*	7,250 ± 523	0	6,100 ± 536	0
KRB	KRB	18,085 ± 312	35,614 ± 1457	96	34,498 ± 728	91
Ca^{++}-free KRB	Ca^{++}-free KRB	11,967 ± 1136*	12,969 ± 1034	8	14,436 ± 1980	20

Each value is the average ± SEM of triplicate determinations. Two separate experiments are shown. Slices from a single dog thyroid were used for each experiment.

* $p < 0.01$ comparing control glucose oxidation in KRB with that in Ca^{++}-free KRB.

AMP (Pastan et al., 1967), the effects of Ca^{++} exclusion from the buffer could be explained in at least two ways. Activation of adenyl cyclase by TSH might require Ca^{++} or alternatively Ca^{++} might be essential for the expression of effects of 3'5' cyclic AMP. The decreased basal glucose oxidation in the Ca^{++}-free buffer could be compatible with either one of these hypotheses. The results summarized in Table II indicate that the more likely explanation was that Ca^{++} was essential for the metabolic effects of 3'5' cyclic AMP. In the absence of Ca^{++}, DBC stimulation of glucose-1-^{14}C oxidation was also markedly decreased, similar to the reduction observed using 1 mu/ml TSH. Furthermore, Rasmussen and Tenenhouse (1968) reported that epinephrine-induced elevation of 3'5' cyclic AMP in rat parotid slices was unimpaired in Ca^{++}-free buffer but that amylase secretion was not increased. We have found that baseline adenyl cyclase activity was the same in thyroid slices whether they had been incubated for two hours in Krebs-Ringer bicarbonate buffer with or without Ca^{++}.

The data in Table III indicate that the addition of Ca^{++} during the final incubation restores basal glucose oxidation and the stimulation produced by 1 mu/ml TSH and 0.5 mg/ml DBC. Thus the results obtained with Ca^{++}-free buffer cannot be attributed to an irreversible or non-specific effect on the thyroid slices.

Ca^{++} was previously reported to be essential for the effects of TSH on increasing thyroid slice weight (Bakke et al., 1957) and ^{131}I transfer from the intrathyroidal pool of iodide into thyroglobulin (Kondo et al., 1963). The results presented in Table IV demonstrate that stimulation of ^{32}P incorporation into phospholipids by small doses of TSH is also dependent upon Ca^{++}. However, in contrast to glucose oxidation, omission of Ca^{++} from the buffer did not consistently reduce basal ^{32}P incorporation. Similar to the experiments measuring $^{14}CO_2$ production, the inhibition caused by omission of Ca^{++} from the buffer was not apparent when larger amounts of TSH were used. TSH responsiveness was again restored when slices which

TABLE III

EFFECT OF ADDITION OF Ca^{++} DURING FINAL INCUBATION

ON TSH AND DBC STIMULATION OF GLUCOSE-1-^{14}C OXIDATION IN DOG THYROID SLICES

Exp. No.	Initial Incubation Buffer	Final Incubation Buffer	$^{14}CO_2$ Produced cpm/gm/45 min.					
			Control	TSH 1 mu/ml	% Stim.	TSH 50 mu/ml	% Stim.	DBC 500 µg/ml	% Stim.
1	KRB	KRB	32,258 ± 1845	56,777 ± 1020	75	93,938 ± 2841	190	48,068 ± 4105	49
	Ca^{++}-free KRB	Ca^{++}-free KRB	30,756 ± 2022	44,245 ± 3321	44	87,482 ± 2993	185	40,985 ± 1584	33
	Ca^{++}-free KRB	Ca^{++}-free KRB	18,873 ± 758*	20,859 ± 1029	11	59,587 ± 5557	217	15,321 ± 756	-19
2	KRB	KRB	20,502 ± 865	47,614 ± 2146	132	99,869 ± 7301	386	57,034 ± 3282	180
	Ca^{++}-free KRB	KRB	19,559 ± 1518	42,332 ± 3162	116	97,601 ± 7782	400	61,524 ± 4225	210
	Ca^{++}-free KRB	Ca^{++}-free KRB	20,426 ± 2649	33,218 ± 3846	62	96,557 + 8529	373	38,529 ± 924	88

Each value is the average ± SEM of triplicate determinations. Two separate experiments are shown. Slices from a single dog thyroid were used for each experiment.

* $p < 0.05$ comparing basal oxidation in KRB with that in Ca^{++}-free KRB.

TABLE IV

EFFECT OF ABSENCE OF Ca^{++} ON TSH STIMULATION OF

^{32}P INCORPORATION INTO PHOSPHOLIPIDS IN DOG THYROID SLICES

Exp. No.	Initial Incubation Buffer	Final Incubation Buffer	^{32}P Incorporated cpm/gm/2 hours					
			Control	TSH 2.5 mu/ml		TSH 10 mu/ml		TSH 100 mu/ml	
					% Stim.		% Stim.		% Stim.
1	KRB	KRB	6,791 ± 652	11,327 ± 277	67	---		16,604 ± 420	145
	Ca^{++}-free KRB	Ca^{++}-free KRB	5,085 ± 324	5,515 ± 313	8	---		9,204 ± 639	81
2	KRB	KRB	7,005 ± 1236	---		11,614 ± 762	66	23,220 ± 1431	231
	Ca^{++}-free KRB	Ca^{++}-free KRB	9,463 ± 580	---		15,121 ± 389	60	22,782 ± 1770	141
	Ca^{++}-free KRB	Ca^{++}-free KRB	7,019 ± 26	---		8,128 ± 1208	16	18,638 ± 2734	166

Each value is the average ± SEM of triplicate determinations. Two separate experiments are shown. Slices from a single dog thyroid were used for each experiment.

had been incubated in Ca^{++}-free buffer were placed in buffer containing Ca^{++} for the final incubation. It is not known whether all the diverse effects of TSH on the thyroid gland are also dependent upon adequate amonts of Ca^{++}. Even if these effects are all mediated via 3'5' cyclic AMP as has been postulated (Pastan et al., 1967), it is conceivable that some of them might be independent of Ca^{++}.

Thus, although these results suggest that Ca^{++} is involved in the expression of the metabolic effects of 3'5' cyclic AMP in the thyroid, the mode or site of this action is not clear. Rasmussen and Tenenhouse (1968) postulated that the primary effect of 3'5' cyclic AMP was to increase the permeability of cellular membranes to Ca^{++}, and it was the intracellular accumulation of this ion which was in fact responsible for the subsequent metabolic effects. The present results would be compatible with this hypothesis since in the absence of extracellular Ca^{++}, DBC or 3'5' cyclic AMP would be without effect. They would also be consistent with a requirement for Ca^{++} for the binding of TSH to the thyroid cell membrane necessary for the activation of adenyl cyclase and for the transport of DBC into the thyroid cell. This latter explanation seems less likely, since epinephrine still increased 3'5' cyclic AMP in rat parotid in the absence of Ca^{++} even though secretion of amylase was not observed (Rasmussen et al., 1968). It is also possible that 3'5' cyclic AMP mediates an intracellular rather than transcellular shift of Ca^{++} or that both 3'5' cyclic AMP and Ca^{++} are essential for the various metabolic effects which have been attributed to the nucleotide. Our preliminary results indicating that increasing the concentration of Ca^{++} in Krebs-Ringer bicarbonate buffer from 1.3 to 11.7 mM in the absence of phosphate stimulates basal glucose oxidation does not clearly distinguish between these various possibilities.

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