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Regulation of Cellular Calcium Metabolism and Calcium Transport by Calcitonin

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Summary. Calcitonin was studied in isolated kidney cells and in isolated mitochondria. A concentration of 10 ng/ml of synthetic calcitonin increases the cellular accumulation of ^{45}Ca and the total cell calcium. The mitochondrial pool is increased several-fold. Kinetic analysis of the data shows that although the total cellular exchangeable calcium pool is enlarged, calcium influx and efflux are significantly depressed by calcitonin. The absence of phosphate or the presence of inhibitors of mitochondrial calcium transport completely abolish the effects of the hormone. In isolated mitochondria, the hormone stimulates the active calcium uptake and depresses the extramitochondrial calcium activity. Calcitonin counteracts the effects of cyclic AMP which stimulates the release of calcium from mitochondria and increases the extramitochondrial calcium activity. These data indicate that cellular calcium homeostasis is controlled by the mitochondrial calcium turnover. They suggest that calcitonin regulates the cell calcium metabolism and inhibits the transcellular calcium transport by stimulating the rate of calcium uptake by mitochondria which depresses cytoplasmic calcium activity.

Effect of Calcitonin on Cellular Calcium Metabolism

In 1969, I reported that porcine calcitonin increases the total calcium content of cultured kidney cells, stimulates the cellular calcium-45 uptake and inhibits calcium efflux from the cells (Borle, 1969). In 1973, Harrell, Binderman and Rodan confirmed these findings in isolated bone cells (Harrell, Binderman & Rodan, 1973). They found that porcine calcitonin enhances the cellular accumulation of calcium while inhibiting the release of radiocalcium from the cells. Both reports concluded that calcitonin may act by inhibiting the active transport mechanism responsible for the cellular extrusion of calcium or by decreasing the concentration of intracellular calcium available for transport. Other indirect evidence suggests that the cytoplasmic calcium activity is depressed by calcitonin (Nisket & Nordin, 1968; MacManus & Whitfield, 1970; Whitfield, MacManus & Gillan, 1971).

Finally, calcitonin has been shown to dramatically increase the number and density of intramitochondrial calcium granules in bone cells (Matthews, Martin, Collins, Kennedy & Powell, 1972).

I have recently proposed that the control and regulation of the cytoplasmic calcium activity depends to a large extent on the exchange of calcium between the mitochondria and the cytoplasm rather than on the transport of calcium across the plasma membrane (Borle, 1973). In light of this new model of cellular calcium homeostasis, I have reinvestigated the cellular effects of calcitonin to test the hypothesis that calcitonin may regulate the cytoplasmic calcium activity by modulation of the mitochondrial controller system. These studies have shown that calcitonin stimulates calcium transport into mitochondria. Thus, by depressing the cytoplasmic calcium activity, calcitonin secondarily inhibits calcium efflux out of the cell and transcellular calcium transport.

Materials and Methods

Cell Cultures

Monkey kidney cells (LLC-MK-2) were grown in culture as a monolayer in MEM (minimum essential medium) and Earl's salt solutions. They were harvested by scraping with a soft rubber policeman and placed in suspension in Krebs-Henseleit buffers. All experiments were done with the cells in suspension.

Calcium Uptake

The cells were placed in a Krebs bicarbonate buffer containing 1.3 mM calcium and 1.0 mM phosphate at pH 7.4. The gas phase consisted of 5% CO_2 in air. After 1 hr of preincubation porcine calcitonin (lyophilized, 12 MRC U/mg, Armour Pharmaceutical Company, Kankakee, Ill.) was added at a concentration of 1 μg/ml along with calcium-45 and the cell suspension incubated for one additional hour. At the end of the incubation, the cells were washed with 10 volumes of ice cold unlabeled buffer, separated by centrifugation, homogenized with an ultrasonic probe. Cell protein, cell calcium, and the cell radioactivity were measured as previously published (Borle, 1969).

Measurements of Intracellular Pools

These measurements were made by prelabeling the cells with calcium-45 for 1 hr followed by isotopic desaturation according to the method previously published (Borle, 1972). These experiments were performed in a Krebs-Henseleit Tris buffer containing 1.3 mM calcium, 1.0 mM phosphate at pH 7.4. Porcine calcitonin 1 μg/ml was added 1 hr before the isotope to assure steady-state conditions.

Measurements of Calcium Influx

These measurements were made in steady-state conditions by the method previously published (Borle, 1970). The suspending medium was a Krebs bicarbonate buffer con-

taining 1.3 mM calcium and 0 or 1 mM phosphate, and the cells preincubated for 2 hr before the experiments. Synthetic salmon calcitonin (Lot No. 5717512 from Sandoz Ltd., Basle, Switzerland) was added at a concentration of 0.01 μg/ml 2 hr before the isotope. Oligomycin and Warfarin were added at a concentration of 10^{-5} M.

Preparation of Mitochondria

Mitochondria were prepared at 4 °C from adult Sprague Dawley rat liver by standard methods as described previously (Borle, 1974*a*). The reaction mixture in which mitochondria were suspended consisted of equal parts of 0.25 M sucrose and of a KCl salt solution. Its final composition was (in mM): 125 sucrose, 5 Na ATP, 10 Na succinate, 4 $MgCl_2$, 4 K_2HPO_4, 10 Tris, 33 KCl. The total osmolarity was 250 mosm. The initial pH was either 7.2 or 7.0. Calcium influx into mitochondria was measured in presence of a calcium-EGTA buffer, at a free calcium concentration of 5 μM (EGTA 1.6 mM, $CaCl_2$ 1.44 mM, pH = 7.0). Perturbations of calcium uptake by cyclic AMP (cAMP) and synthetic salmon calcitonin (SCT) were performed without calcium-EGTA buffer to allow the medium calcium to fluctuate. Calcium-45 (2 μCi/ml) was added to the mitochondrial suspension at time zero and since the specific activity of the isotope was 10 mCi/mg, the total calcium concentration of the medium was 5 μM. One-ml aliquots of the mitochondrial suspension were taken at appropriate times with an Eppendorf pipette and the medium was immediately separated from the mitochondria by filtration through 0.45 μ millipore filters. The radioactivity of the medium was measured by liquid scintillation spectrophotometry. The radioactivity of the mitochondria was measured after washing the filter with ice cold unlabeled medium, and placing the filter in the scintillation vials. All measurements of calcium-45 were made in Aquasol (New England Nuclear, Boston, Mass.) on an L-100 Beckman liquid scintillation spectrophotometer.

Results

Total Calcium and Calcium Uptake

Table 1 shows that 1 hr after addition of 1 μg/ml calcitonin the total cell calcium has increased from 20.3 to 26.6 nmoles/mg cell protein. Calcium uptake also increases 30% from 8.18 to 10.7 nmoles/mg protein × hr. The absolute values of calcium uptake are larger than those obtained previously

Table 1. Effect of 1 μg/ml porcine calcitonin on total cell calcium and calcium-45 uptake in isolated kidney cells

	Total cell Ca (nmoles/mg prot)	Calcium uptake (nmoles/mg prot × hr)	% Exchange
Control (6)	20.3 ± 0.7	8.18 ± 0.25	40.4 ± 1.4
Calcitonin, 1 μg/ml (6)	26.6 ± 1.63	10.69 ± 0.26	40.9 ± 2.9
% increase	+31%	+31%	N.S.
p value	<0.01	<0.001	N.S.

Values are the mean ± SE of 6 experiments. N.S. = not significantly different.

(Borle, 1969) because these experiments are made with cell suspensions and not with cell monolayers. The surface area available for exchange and the rate of stirring are much greater in cell suspensions than in cell monolayers. On the other hand, the increased calcium uptake produced by calcitonin is less than that reported in the previous paper (Borle, 1969), because the hormone was added at time 0, along with calcium-45. These are not steady-state conditions. In the first report, calcitonin was added during the preincubation period 1 hr before the addition of the isotope. It is not possible to decide by this experiment alone whether calcitonin increases calcium influx, inhibits calcium efflux or increases the intracellular exchangeable pool of calcium. However, the fact that the relative specific activity of the cell, expressed as % exchange, is not affected, suggests that the larger total calcium and the increased uptake are due at least in part to an inhibition of calcium transport out of the cell.

Distribution of Intracellular Calcium

In a recent paper, I have shown by kinetic analysis of calcium-45 desaturation curves that intracellular calcium was divided into two distinct exchangeable compartments (Borle, 1972). The slowest compartment can be identified as a mitochondrial calcium pool while the second represents the cytoplasmic calcium pool. The evidence for the identification of both pools has already been published (Borle, 1972). Using the same technique, I investigated the effects of calcitonin on the steady-state distribution of intracellular calcium. In these experiments, porcine calcitonin was used at a concentration of 1 μg/ml. Table 2 shows that, after 1 hr of exposure to calcitonin, the cytoplasmic pool was slightly enlarged but more significantly the mitochondrial calcium pool increased 10-fold. This could be due to an increased calcium uptake by mitochondria, to the subsequent sequestration of calcium in the

Table 2. Effect of 1 μg/ml porcine calcitonin on the intracellular calcium pools of isolated kidney cells determined by calcium-45 desaturation

	Cytoplasmic calcium pool (pool 2) (nmoles/mg prot)	Mitochondrial calcium pool (pool 3) (nmoles/mg prot)
Control (19)	1.11 ± 0.06	0.68 ± 0.05
Calcitonin, 1 μg/ml (6)	1.54 ± 0.13	7.32 ± 1.7
% increase	+39%	+976%
p value	<0.01	<0.001

Values are mean ± SE. The number of experiments are indicated in parentheses.

mitochondrial pool or to an inhibition of calcium release from mitochondria. The small increase in the cytoplasmic pool does not mean that the concentration of ionized calcium in the cytoplasm is increased. The concentration of free calcium in the cytoplasm is probably less than 1% of the exchangeable calcium pool (Borle, *unpublished observation*). In addition, with a 10-fold increase in the third kinetic phase of the calcium desaturation curve, it is possible that the size of the second kinetic phase may be spuriously affected and overestimated by our calculations. In any case, these experiments clearly show that one of the actions of calcitonin occurs at the mitochondrial level.

Effect of Synthetic Calcitonin on Calcium-45 Uptake Curves

Since calcitonin increases the sequestration of calcium in mitochondria, the hormone could possibly stimulate the mitochondrial uptake of calcium. Two conditions are required for a significant accumulation of calcium by mitochondria: 1) the presence of phosphate (Lehninger, Carafoli & Rossi, 1967; Lehninger, 1970; Carafoli & Lehninger, 1971) and 2) the availability of ATP or of substrates to provide energy for calcium transport. In absence of either condition, the accumulation of calcium in the mitochondrial pool of isolated cells does not occur (Borle, 1972). Consequently, if calcitonin stimulates calcium uptake into mitochondria, the absence of phosphate or the presence of mitochondrial inhibitiors of calcium uptake should inhibit the effects of the hormone. To test this hypothesis, I studied the effects of synthetic calcitonin on the cellular uptake of calcium-45 in the presence and in the absence of phosphate and of mitochondrial inhibitors. The concentration of phosphate in the incubating medium was either 0 or 1 mM. Two inhibitors were used together to inhibit mitochondrial calcium uptake: 10^{-5} M oligomycin to inhibit the ATP supported calcium uptake and 10^{-5} M Warfarin to inhibit the substrate supported calcium accumulation.

Fig. 1 shows that 0.01 μg/ml of synthetic salmon calcitonin significantly increases the uptake of calcium-45 by isolated kidney cells in presence of 1 mM phosphate. Fig. 2 shows that without phosphate the same concentration of calcitonin fails to increase calcium-45 uptake. Similarly, Fig. 3 shows that inhibitors of mitochondrial calcium uptake totally suppress the effects of calcitonin, even with a normal medium phosphate concentration.

In each case, calcium uptake can be described by a double exponential equation. In all figures, the open and closed circles represented the mean ± the standard error of six experimental determinations. The uptake curve superposed on the experimental data is reconstructed by computer from the coefficients and exponential constants obtained by graphical analysis

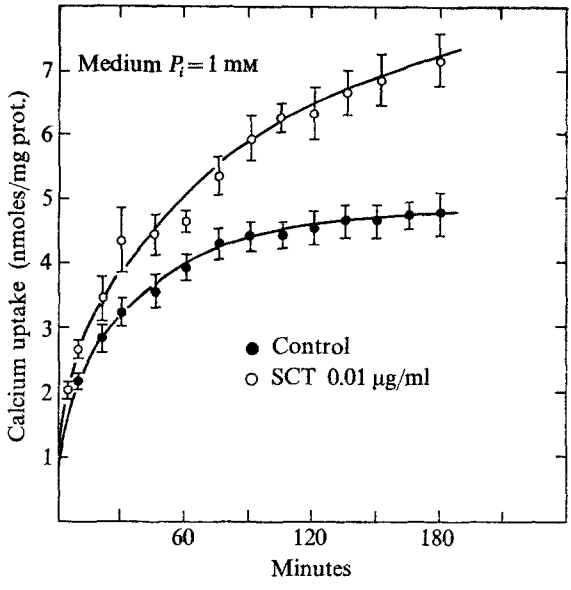

Fig. 1. Effect of synthetic salmon calcitonin on calcium-45 uptake by isolated kidney cells with a medium phosphate of 1.0 mM. Calcitonin, 0.01 μg/ml, was added 2 hr before calcium-45. The points are the mean ± SE of 6 determinations. The lines were generated by computer from the constants and exponential coefficients shown in Table 3

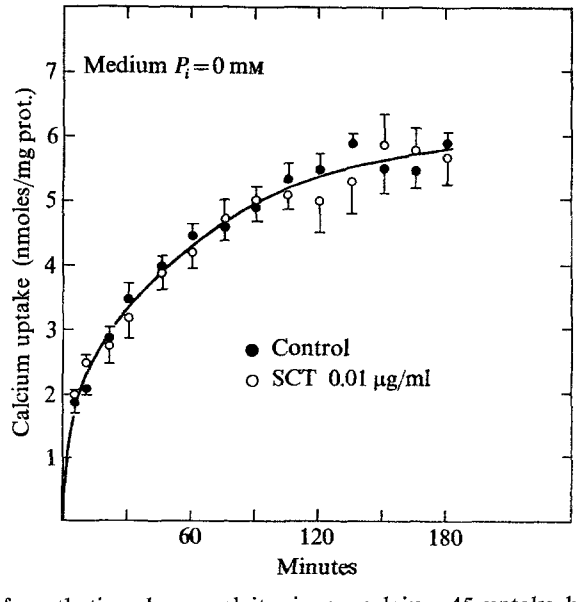

Fig. 2. Effect of synthetic salmon calcitonin on calcium-45 uptake by isolated kidney cells, in absence of extracellular phosphate. Calcitonin, 0.01 μg/ml, was added 2 hr before calcium-45. The points are the mean ± SE of 6 determinations. The lines were generated by computer from the constants and exponential coefficients shown in Table 3

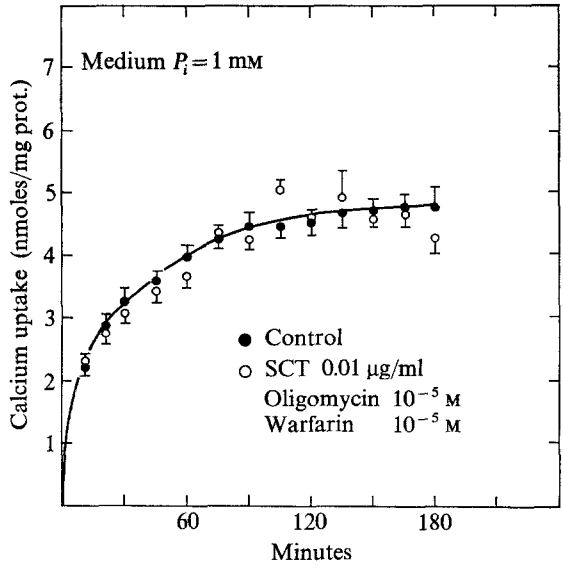

Fig. 3. Effect of synthetic salmon calcitonin on calcium-45 uptake by isolated kidney cells in presence of 1 mM phosphate and 10^{-5} M oligomycin and Warfarin. Calcitonin, 0.01 µg/ml, was added 2 hr before calcium-45. The points are the mean ± SE of 6 determinations. The lines were generated by computer from the constants and exponential coefficients shown in Table 3

Table 3. Coefficients and exponential constants of the double exponential equations describing the calcium uptake curves obtained in different experimental conditions

Experimental conditions	A	B	λ_a	λ_b
Control, 1 mM phosphate	950	88	0.630	0.026
SCT 0.01 µg/ml, 1 mM phosphate	1150	70[a]	0.550	0.012[a]
Control, no phosphate	900	92	0.600	0.022
SCT 0.01 µg/ml, no phosphate	900	90	0.650	0.022
Inactivated SCT	800	80	0.670	0.025
SCT 0.01 µg/ml, 1 mM phosphate + 10^{-5} M oligomycin and Warfarin	1000	80	0.600	0.020

[a] $p < 0.01$.

of the data. Table 3 presents the fast and slow phase coefficients A and B and the fast and slow phase exponential constants λ_a and λ_b of each curve. In each case, the fast phase coefficients and exponential constants are not statistically different from control. The only significant change is seen in the slow phase of uptake obtained with 0.01 µg/ml calcitonin in presence of 1 mM phosphate.

Table 4. Statistical significance of difference between the coefficients and exponential constants of control and experimental curves

	A	B	λ_a	λ_b
Control 1 mM phosphate				
Combined curve	950	88	0.630	0.026
Sum of individual curves	916 $\pm$46	85.6$\pm$2.4	0.620$\pm$0.026	0.025 $\pm$0.002
Calcitonin 0.01 μg/ml, 1 mM phosphate				
Combined curve	1150	70	0.550	0.0120
Sum of individual curves	1033$\pm$82	71 $\pm$3.0	0.520$\pm$0.02	0.0127$\pm$0.0007
Difference	+13%	−17%	−16%	−50%
Significance	N.S.	$p<0.01$	N.S.	$p<0.01$

To assess the statistical significance of the results, each individual curve must be analyzed separately to obtain the exponential parameters of each experiment. Table 4 shows that there is very little difference between the parameters of the combined curve graphed in the figures and the mean of the parameters obtained from individual curves in control and experimental conditions. The only statistically significant findings are a 17% drop in the slow phase coefficient B and a 50% decrease of the slow phase exponential constant λ_b in the experimental group.

Calculation of Calcium Fluxes and Calcium Compartment Sizes

To calculate the calcium fluxes and the compartment sizes from the coefficients and exponential constants, the parallel and the series case of a three-compartment system were considered. Since the time constants of the two phases differ by more than one order of magnitude, one would expect no significant difference between the parallel and series case (Borle, 1970, 1973, 1974*b*). The effects of 0.01 μg/ml calcitonin with 1 mM phosphate are shown in Tables 5 and 6. There is indeed no significant difference between parallel and series systems. Calcitonin produces minor changes in the fast phase, but none are statistically significant (Table 5). This fast phase probably represents calcium binding to the cell surface (Borle, 1970). On the other hand, Table 6 shows that calcitonin markedly influences every parameter of the slow phase which represents calcium exchange between the intracellular exchangeable calcium pools and the cell environment (Borle, 1970, 1972). All effects are statistically significant. First, calcitonin increases the intracellular calcium pool 65%. Second, calcium exchange between the

Table 5. Effects of 0.01 μg/ml of synthetic salmon calcitonin on the fast phase calcium flux and extracellular exchangeable calcium pool

	Control	SCT
A. Parallel system		
Fast phase calcium flux (pmoles mg prot^{-1} min^{-1})	916 ±46	1033 ±68
Extracellular calcium pool (nmoles mg prot^{-1})	1.51 ±0.16	2.01 ±0.20
Efflux rate constant k_{21} (min^{-1})	0.625±0.026	0.516±0.017
B. Series system		
Fast phase calcium flux (pmoles mg prot^{-1} min^{-1})	1002 ±47	1104 ±69
Extracellular calcium pool (nmoles mg prot^{-1})	1.80 ±0.17	2.30 ±0.21
Efflux rate constant k_{21} (min^{-1})	0.568±0.023	0.484±0.015

Data calculated from the calcium uptake curve of Fig. 1.

Table 6. Effects of 0.01 μg/ml of synthetic salmon calcitonin on the slow phase calcium flux and on the intracellular exchangeable calcium pool

	Control	SCT
A. Parallel system		
Slow phase calcium flux (pmoles mg prot^{-1} min^{-1})	85.6 ±2.4	71.0 ±2.4[a]
Intracellular calcium pool (nmoles mg prot^{-1})	3.49 ±0.29	5.66 ±0.41[b]
Efflux rate constant k_{31} (min^{-1})	0.0252±0.0013	0.0127±0.0006[b]
B. Series system		
Slow phase calcium flux (pmoles mg prot^{-1} min^{-1})	85 ±2.8	72 ±2.6[a]
Intracellular calcium pool (nmoles mg prot^{-1})	3.21 ±0.29	5.37 ±0.40[b]
Efflux rate constant k_{32} (min^{-1})	0.0272±0.0018	0.0135±0.0006[b]
Influx rate constant k_{23} (min^{-1})	0.0494±0.0095	0.0319±0.0022[a]

Data calculated by computer from the calcium uptake curve of Fig. 1.

[a] $p<0.05$.

[b] $p<0.01$.

Table 7. Slow phase calcium fluxes, intracellular calcium pools and efflux rate constants calculated for a parallel system from the calcium uptake curves obtained in different experimental conditions

Experimental conditions	Slow phase Ca flux (pmoles mg prot^{-1} min^{-1})	Intracellular Ca pool (nmoles mg prot^{-1})	Efflux constant (min^{-1})
Control, 1 mM phosphate	85	3.42	0.025
SCT 0.01 μg/ml, 1 mM phosphate	70[a]	5.83[a]	0.012[a]
Control, no phosphate	92	4.18	0.022
SCT 0.01 μg/ml, no phosphate	90	4.09	0.022
Inactivated SCT	80	3.20	0.025
SCT 0.01 μg/ml, 1 mM phosphate $+10^{-5}$ M oligomycin and Warfarin	80	4.00	0.020

[a] $p < 0.01$ compared with control 1 mM phosphate.

cell and its environment is depressed 16% by calcitonin. Since the cell is at steady state, both influx and efflux are inhibited by the hormone. Third, calcitonin depresses the efflux rate constant 50%. Finally, when the series system is considered, the influx rate constant k_{23} from the extracellular to the intracellular calcium pool is also depressed 35%. These results confirm the findings reported earlier in this paper and those previously published (Borle, 1969; Harrell *et al.*, 1973).

The effects of calcitonin on all these parameters are totally suppressed by inhibitors of mitochondrial calcium uptake and in the absence of phosphate. Table 7 presents the results obtained in different experimental conditions. Fast and slow phase parameters were calculated for both the parallel and the series case, but since calcitonin has no effect on the fast phase and since there is no difference between both cases, only the slow phase of the parallel case is presented for simplicity. The absence of phosphate slightly increases calcium exchange in control cells which could be expected (Borle, 1972, 1973), but the change is not statistically significant. Without phosphate or in the presence of mitochondrial inhibitors, however, calcitonin has no effect on any of the three parameters.

Effect of Calcitonin on Calcium Uptake in Isolated Mitochondria

Although no one knows whether calcitonin enters the cells, it is not impossible for a polypeptide hormone to be found in mitochondria (Nordquist & Palmieri, 1974). Since I have shown that cyclic AMP has a dramatic

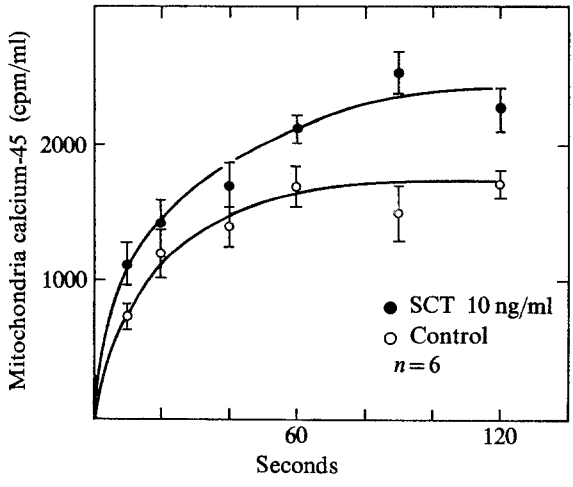

Fig. 4. Effect of synthetic salmon calcitonin on calcium-45 uptake by isolated liver mitochondria. Experimental conditions: 0.5 to 0.8 mg protein/ml; total volume 15 ml; ATP = 5 mM; Na succinate 10 mM; Tris 10 mM; sucrose 125 mM; KCl to 250 mosmoles; Mg 4 mM; P_i 4 mM; initial pH 7.0. Free calcium concentration buffered at 5 μM with EGTA (EGTA 1.6 mM, $CaCl_2$ 1.44 mM). 2 μCi/ml calcium-45 and 10 ng/ml calcitonin added at time 0. Each point is the mean ± SE of 6 determinations

effect on the calcium turnover of liver mitochondria (Borle, 1974*a*), I decided to test the effects of synthetic salmon calcitonin on the same subcellular system.

Fig. 4 shows that 0.01 μg/ml calcitonin enhances the uptake of calcium-45 by mitochondria. In these experiments the concentration of free calcium in the suspending medium was maintained constant at 5×10^{-6} M with a calcium EGTA buffer. This effect is extremely fast: calcitonin being added at time 0 with calcium-45. Calcium uptake rises 50% ($p < 0.01$) within 10 sec. This increased calcium-45 uptake is probably caused by an increased calcium influx, and by an increased sequestration of calcium in the mitochondria. If calcitonin enhances calcium influx, it should counteract the effects of cyclic AMP which stimulates calcium release from mitochondria and increases the steady-state concentration of calcium in the suspending medium (Borle, 1974). Fig. 5 shows the activity of calcium-45 in the mitochondrial suspending medium in absence of a calcium EGTA buffer and with an initial calcium concentration of 5 μM. Five minutes after the addition of the isotope, 3×10^{-6} M cyclic AMP added to the suspension produces an immediate release of isotope into the medium. Calcitonin added 2 min later causes an immediate 40% drop in the medium radioactivity ($p < 0.02$). The time lapse between addition of calcitonin and sampling of the medium

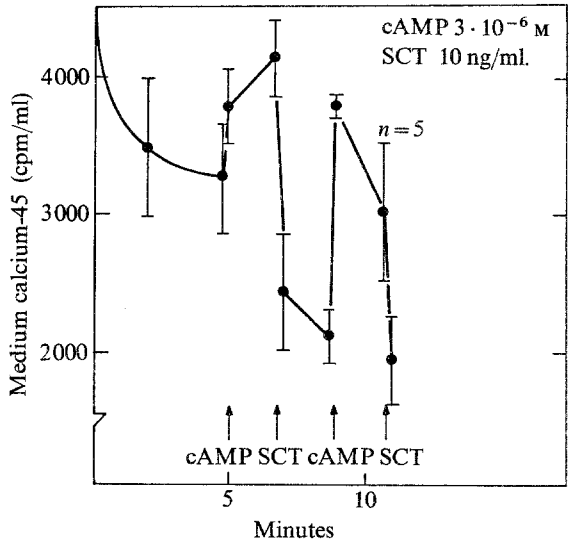

Fig. 5. Effects of cyclic AMP and of synthetic salmon calcitonin on the radioactivity of the medium bathing isolated liver mitochondria. Experimental conditions same as in Fig. 4 except that calcium was not buffered by EGTA. Initial calcium concentration = 5 μM. 2 μCi/ml calcium-45 added at time 0. Cyclic AMP, 3×10^{-6} M added at 4.75 and 8.75 min. Calcitonin, 10 ng/ml, added at 6.75 and 10.75 min. Each point is the mean ± SE of 5 determinations

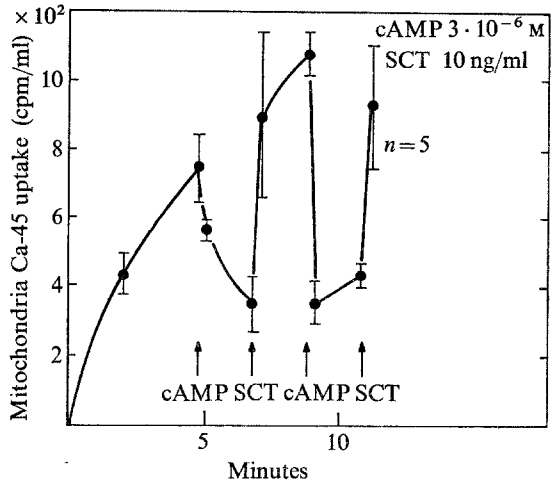

Fig. 6. Effects of cyclic AMP and synthetic salmon calcitonin on calcium-45 uptake by liver mitochondria. Experimental conditions same as in Fig. 5

was 5 sec. Later addition of cyclic AMP within seconds doubles the medium activity ($p < 0.01$). Further addition of calcitonin again reduces the isotope concentration of the suspending medium. Fig. 6 shows that the radioactivity

of calcium-45 in the mitochondria presents the mirror image of the suspending medium activity. These experiments suggest that calcitonin stimulates the rate of uptake of calcium into mitochondria. As a result, a new steady state is established, with a lower calcium activity in the mitochondrial environment.

Because of the very fast turnover of calcium between the mitochondria and their suspending milieu, the effects of calcitonin and cyclic AMP on calcium influx and efflux are very rapidly reflected in the steady-state levels of calcium in and out of the mitochondria.

Discussion

Many investigators have shown that calcitonin depresses calcium transport in bone, intestine and in the kidney (Friedman & Raisz, 1965; Aliapoulios, Goldhaber & Munson, 1966; Pechet, Bobadilla, Carroll & Hesse, 1967; Friedman, Au & Raisz, 1968; Pak, Ruskin & Casper, 1970; Barlet, 1972; Barlet & Care, 1972; Olson, DeLuca & Potts, 1972; Swaminathan, Ker & Care, 1974). The evidence presented here supports the idea that calcitonin interferes with the transcellular transport of calcium.

The data presented in Table 1 confirm that calcitonin increases the total cell calcium concentration (Borle, 1969; Harrell *et al.*, 1973). Table 2 shows that most, if not all, of the calcium accumulated by the cells is found in a kinetic compartment that we have identifed as a mitochondrial pool (Borle, 1972). These results agree with the electron-micrographic studies of Matthews and collaborators who reported a very large increase in the formation of mitochondrial calcium phosphate granules in bone cells treated with calcitonin (Matthews *et al.*, 1972). The increased mitochondrial pool of calcium observed by isotopic desaturation, 6.4 nmoles/mg protein, can fully account for the rise in total cellular calcium concentration, 6.3 nmoles/mg protein (Tables 1 and 2). The small rise in the cytoplasmic exchangeable calcium pool, although statistically significant, is probably spurious. The 10-fold expansion of the third kinetic phase of the calcium desaturation curve is so large that it probably influences the calculations of the second kinetic compartment.

Fig. 1 shows that calcitonin stimulates calcium-45 uptake by isolated cells. The hormone does not affect the fast phase of exchange with the extracellular exchangeable calcium pool (Table 5). However, each parameter of the slow phase is significantly affected by calcitonin (Table 6). This phase is assumed to reflect the exchange of calcium between extracellular fluids and all intracellular pools (Borle, 1969, 1970, 1972). The

analysis of the data reveals several important points. First, the analysis of the uptake data as a parallel or as a series system gives practically identical results, as observed previously (Borle 1970, 1974*b*). Second, despite an increased calcium-45 uptake, the actual calcium fluxes are depressed by calcitonin. This apparent paradox underlines the difficulty in interpreting an isotope uptake curve. Indeed, when the system is at steady state, an increased uptake of isotope may reflect an increased exchangeable pool with a higher, a normal or a decreased influx or an increased influx with a higher, a normal or a depressed calcium pool. Third, since the system is at steady state, influx equals efflux. Consequently, calcitonin inhibits both calcium influx into the cell and calcium efflux out of the cell. Fourth, the total intracellular exchangeable calcium pool is increased 60 to 70% and is totally responsible for the greater calcium-45 uptake. Although the results obtained in uptake and in desaturation experiments agree qualitatively, there is an obvious quantitative discrepancy: in control conditions, the second phase of uptake which we assume to represent the total intracellular exchangeable calcium pool, is slightly less than twice the sum of compartments 2 and 3 of desaturation which we assume to be the cytoplasmic and mitochondrial pool, respectively (Borle, 1972). Obviously, the identification of these pools is an oversimplification. In experimental conditions, the second phase of uptake is 35 to 40% smaller than phases 2 and 3 of desaturation. Of course, both types of experiments, differ on one important point: uptake is measured in a closed and desaturation in an open system. However, the most likely explanation is that in uptake experiments, treating the data as a parallel system gives results mathematically identical to a series system (Tables 4, 5 and 6). In desaturation experiments, on the other hand, this leads to an overestimation of the third compartment (Huxley, 1960). A literal and quantitative comparison of both types of data is, therefore, impossible and one should consider only the qualitative aspect of these effects of calcitonin. Fifth, the apparent efflux rate constant is depressed 50%. The actual efflux rate constant cannot be calculated since the cytoplasmic concentration of free calcium is not known. Nevertheless, these results confirm the conclusions that calcitonin inhibits calcium efflux out of the cell (Borle, 1969; Harrell *et al.*, 1973).

Theoretically, calcitonin could inhibit an active transport mechanism in the plasma membrane, or it could decrease the substrate available for transport, i.e. the cytoplasmic calcium activity. I have recently proposed that the concentration of free calcium in the cytosol is likely to be regulated by the mitochondria to a much greater extent than by the transport of calcium in and out of the cell across the plasma membrane (Borle, 1972, 1973). If this

model is correct, calcitonin could very well affect calcium transport in mitochondria. For instance, the accumulation of calcium into mitochondria induced by calcitonin (Table 2), could be due to a stimulation of mitochondrial calcium uptake or to a decreased mitochondrial release of calcium. To test this hypothesis, I took advantage of the fact that calcium uptake by isolated mitochondria is stimulated by phosphate and depressed by specific mitochondrial inhibitors such as oligomycin and Warfarin (Lehninger *et al.*, 1967; Lehninger, 1970; Carafoli & Lehninger, 1971). The same effects have been observed in whole cells by kinetic analysis of calcium-45 desaturation curves (Borle, 1972). The experiments shown in Figs. 2 and 3 indicate that calcitonin activity requires the presence of phosphate and of an intact mitochondrial calcium transport mechanism. Table 3 shows that, in absence of phosphate in the extracellular fluids, the kinetic parameters of the calcium-45 uptake curve obtained with 0.01 μg/ml calcitonin are identical to the control curve without phosphate. Consequently, the calcium flux, the calcium pool, and the efflux rate constant show no difference between the control and experimental groups, in absence of phosphate (Table 7). As one could predict, the parameters of the fast phase in a parallel and in a series system show no difference, and the results are not presented for brevity.

The presence of oligomycin and Warfarin also abolishes the effects of calcitonin (Fig. 3, Tables 3 and 7), indicating again that calcitonin activity requires an intact mitochondrial transport system. Consequently, one can postulate that calcitonin acts by stimulating calcium transport into mitochondria because 1) calcitonin increases the sequestration of calcium in the mitochondrial pool, 2) its activity requires the presence of phosphate, and 3) it is abolished by inhibitors of mitochondrial calcium uptake.

To further test this hypothesis, the effects of calcitonin were studied in isolated mitochondria. It is, of course, impossible to know whether calcitonin penetrates the cell membrane in physiological conditions and whether its receptor site is located inside the cell. However, the transport of a polypeptide hormone across a cell membrane and its intracellular localization *in vivo* is not impossible. Nordquist and Palmieri (1974) have shown that tritiated or ^{125}iodinated parathyroid hormone (PTH) can be localized in the cytoplasm of renal cells of the proximal tubule. If PTH can really cross the cell membrane, calcitonin could very well do so. The effects of synthetic calcitonin on calcium uptake by isolated mitochondria are extremely fast. When the calcium activity of the suspending medium is maintained constant at 5 μM with a Ca EGTA buffer, calcium uptake by mitochondria is significantly increased 10 sec after the addition of calcitonin (Fig. 4). This clearly

suggests that calcitonin stimulates the rate constant of calcium influx into mitochondria. Since the hormone and the isotope were added at the same time, at the beginning of the experiment, we can exclude the possibility that the increased calcium-45 uptake is due to a decreased calcium efflux. The shift of calcium-45 into mitochondria, produced by calcitonin, is extremely rapid and occurs in less than 5 sec (Fig. 6). There is a concomitant and equally rapid fall in calcium-45 activity in the medium (Fig. 5). These results again indicate that calcitonin stimulates calcium influx into mitochondria. The rapidity of the calcitonin action is due to the extremely fast turnover of calcium between the mitochondrial matrix and the medium.

These results obtained in isolated mitochondria fit perfectly with those obtained in intact cells. They clearly support the model of cellular calcium homeostasis and of its regulation by calcitonin, which I recently proposed (Borle, 1973). This model predicts that intracellular calcium metabolism and calcium transport is controlled by the mitochondria and regulated by various hormones and ions through their actions on the mitochondrial calcium turnover. At steady state, calcium influx from cytoplasm to mitochondria, J_{cm}, must equal calcium efflux from mitochondria to cytoplasm, J_{mc}:

$$J_{cm}=J_{mc}, \tag{1}$$

$$J_{cm}=k_{cm}\cdot \mathrm{Ca}_c, \tag{2}$$

$$J_{mc}=k_{mc}\cdot \mathrm{Ca}_m. \tag{3}$$

The cytoplasmic calcium activity, Ca_c is thus a function of three parameters (Borle, 1973):

$$\mathrm{Ca}_c=\frac{\mathrm{Ca}_m\cdot k_{mc}}{k_{cm}} \tag{4}$$

where Ca_m is the mitochondrial calcium activity, k_{mc} the rate constant of calcium efflux from mitochondria to cytoplasm, and k_{cm} the rate constant of calcium influx from cytoplasm to mitochondria. Since the mitochondrial calcium activity, Ca_m, is in equilibrium with calcium phosphate precipitates present in the mitochondrial matrix, it will be fixed by the solubility product constant K_{sp} of the particular form of calcium phosphate:

$$\mathrm{Ca}_m\cdot \mathrm{P}_i=K_{sp}, \tag{5}$$

$$\mathrm{Ca}_m=\frac{K_{sp}}{\mathrm{P}_i}. \tag{6}$$

Since the mitochondrial calcium activity will be a function of the K_{sp} and inversely proportional to the concentration of inorganic phosphate, P_i, we can write:

$$Ca_c = \frac{Ca_m \left(f \frac{K_{sp}}{P_i} \right) k_{mc}}{k_{cm}}. \tag{7}$$

The model further proposes that cytoplasmic calcium activity determines calcium efflux out of the cell by increasing or decreasing the concentration of substrate available to the active transport mechanism. In addition, the cytoplasmic calcium activity also regulates the membrane permeability to calcium: an increased cytoplasmic calcium activity stimulates calcium influx, a decreased activity depresses calcium influx into the cell (Borle, *in preparation*).

All the data presented in this paper are consistent with the idea that calcitonin stimulates the rate constant of calcium influx into mitochondria, k_{cm}. If this hypothesis is correct the model predicts that the consequences of this single effect will be as follows: 1) calcium uptake into mitochondria increases; 2) precipitation of calcium phosphate in the mitochondrial matrix takes place without changing the calcium activity in the mitochondria which is fixed by the solubility product of the mineral phase; 3) according to Eq. (7) the rise in k_{cm} lowers the cytoplasmic calcium activity Ca_c; 4) the fall in cytoplasmic calcium activity reduces the efflux of calcium out of the cell; 5) the fall in cytoplasmic calcium activity also depresses calcium influx from the extracellular fluids to the cell interior; 6) the depression of calcium transport in and out of the cell results in a decreased transcellular transport of calcium; 7) despite the depression of cellular calcium transport, and despite a lower cytoplasmic calcium activity, the accumulation of calcium in the mitochondria produces an increase in the total cell calcium; 8) a lowering of the extracellular phosphate concentration, or its absence, lowers the intracellular phosphate concentration and depresses the accumulation of calcium and phosphate in the mitochondria. Since the mitochondrial calcium activity is a function of both the K_{sp} and the phosphate concentration, Ca_m will rise in these conditions, counteracting the effect of calcitonin on k_{cm} in the denominator of Eq. (7); this would explain the suppression of the effects of calcitonin in absence of phosphate; 9) specific inhibitors of calcium uptake into mitochondria inhibit k_{cm} and thereby abolish the effects of calcitonin; 10) an increased concentration of phosphate in the extracellular fluids will increase the intracellular phosphate concentration and the mitochondrial uptake of calcium and phosphate. Moreover, since the mito-

chondrial phosphate concentration depresses the mitochondrial calcium activity Ca_m, it will enhance the effects of calcitonin by further lowering the cytoplasmic calcium activity Ca_c. Admittedly, the cytoplasmic calcium activity of living cells cannot be measured directly and the effects of calcitonin on the free calcium concentration of the cytosol must remain speculative. However, such an action has already been proposed by several investigators (Borle, 1969; MacManus & Whitfield, 1970; Whitfield *et al.*, 1971; Harrell *et al.*, 1973). Our interpretation of the data relies heavily on the assumptions underlying our proposed model of mitochondrial control of cellular calcium homeostasis. These assumptions are: 1) that mitochondrial calcium exchange is the main determinant of cytoplasmic calcium activity; 2) that cytoplasmic calcium activity determines calcium exchange and calcium transport across the plasma membrane; and 3) that the regulation of the cellular calcium homeostasis and cellular calcium transport takes place at the mitochondrial level.

All the effects of calcitonin and phosphate observed in this paper have been obtained experimentally. The only report which conflicts with these results and with the proposed model, appeared in the proceedings of the Second International Symposium on Calcitonin (Parkinson & Radde, 1970). However, this short communication, consisting of six experiments, has never been confirmed or duplicated. Moreover, the system used, the red blood cell ghosts, is not a good model for epithelial or mesenchymal cells since erythrocytes have no mitochondria. Finally, the conditions used make the data difficult to interpret because calcitonin is shown to have no effect in presence of ATP, and because the reported increase in calcium efflux is not accompanied by an increased ATPase activity. Although it is often claimed that calcitonin stimulates calcium efflux out of the cell (Rasmussen, 1971; Rasmussen, Kurokawa & DeLong, 1971; Copp, 1973; Rasmussen & Bordier, 1974), the data presented here and other published results clearly show that cellular calcium efflux is depressed by calcitonin (Borle, 1969; Harrell *et al.*, 1973).

The enhancement of the effects of calcitonin by phosphate is also well documented (Hirsch, 1968; Kennedy, Tanzer & Talmage, 1969; Orimo, Fujita & Yoshikawa, 1969; Raisz & Niemann, 1969; Wener, Gorton & Raisz, 1972; Hirsch, Sliwowski, Orimo, Darago & Mewborn, 1973). This model can clearly account for this effect, but it further predicts that calcitonin activity should be depressed or even abolished in absence of phosphate.

Several investigators have reported that calcitonin increases the cellular concentration of cyclic AMP in bone and kidney (Melson, Chase & Aurbach,

1970; Murad, Brewer & Vaughan, 1970; Heersche, Marcus & Aurbach, 1974; Rodan & Rodan, 1974), and we have recently demonstrated that cyclic AMP stimulates calcium efflux from mitochondria and increases the extramitochondrial calcium activity (Borle, 1974*a*). Figs. 5 and 6 clearly show that calcitonin counteracts the effects of cyclic AMP and vice versa. How can we account for this paradox? It has already been suggested that although both calcitonin and parathyroid hormone increase the cellular concentration of cyclic AMP, they act on separate, independent sites (Heersche *et al.*, 1974; Rodan & Rodan, 1974). It has also been shown that calcium inhibits adenyl cyclase in bone, heart muscle, fat and renal cortex (Birnbaumer, Pohl & Rodbell, 1969; Chase, Fedak & Aurbach, 1969; Streeto, 1969; Drummond & Duncan, 1970; Marcus & Aurbach, 1971). However, since the half-maximal inhibition, K_i, lies between 10^{-4} and 10^{-3} M, the physiological significance of this finding is presently doubtful. On the other hand, adenyl cyclase of parathyroid tissue is inhibited by calcium with a K_i of 5×10^{-6} M (Dufresne & Gitelman, 1972) which is in the range of the postulated intracellular concentration of free calcium. It is clear that more work is needed to precisely assess the interaction between calcium and adenyl cyclase activity and the existence of a possible negative feedback loop between the enzyme, cyclic AMP and intracellular calcium. Nevertheless, one could postulate that the increased cyclic AMP concentrations observed after calcitonin administration may be due to a decreased cytoplasmic calcium, resulting in a derepression of adenyl cyclase activity.

Finally, can we relate these effects of calcitonin obtained in isolated kidney cells to the systemic actions of the hormone? To do that, one has to assume that the cellular mode of action of calcitonin is identical in bone, in intestine and in the kidney. The fact that Harrell *et al.* (1973) have found that the effects of calcitonin on bone cells are practically identical to those obtained in kidney cells is encouraging. We have to assume also that the plasma concentration of calcium is controlled and regulated by the steady-state exchange of calcium between the extracellular fluids on one hand and the bone fluids, the intestinal lumen and the renal tubular fluids on the other. This exchange is presumably under the control of the cellular transport of calcium.

In conclusion, these experiments performed in isolated cells suggest that calcitonin depresses the cytoplasmic calcium activity and the cellular transport of calcium by regulating the mitochondrial calcium controller mechanism. Such an action could lead to a decreased rate of calcium transport and to the hypocalcemia observed in experimental animals.

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