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EFFECT OF CALCIUM CHANNEL AGONIST BAY K 8644 ON CALCITONIN SECRETION FROM A RAT C-CELL LINE

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Summary: Bay K 8644, a novel dihydropyridine, stimulates calcitonin secretion in a dose-dependent manner from a rat medullary thyroid carcinoma cell line, rMTC 6-23, and causes an increase in cytosolic free calcium concentration, as measured by quin-2. These effects are competitively inhibited by nifedipine, and completely abolished in the absence of extracellular calcium. These data suggest that calcium influx via voltage-dependent calcium channels plays a crucial role in the regulation of cytosolic free calcium concentration and calcitonin secretion.

Dihydropyridines, such as nifedipine, nitrendipine, and nimodipine, are assumed to block the entry of Ca into the cells at the voltage-dependent Ca^{++} channels (1). Recently, Bay K 8644, a nifedipine analogue, has been reported to act as a Ca^{++} channel agonist, rather than an antagonist, in a variety of tissues and cells, the heart (2-6), the smooth muscle (2), adrenal chromaffin cell (7), pituitary cell (8), and pancreatic B-cell (9).

It is widely accepted that Ca^{++} is an important messenger of the various cellular functions. A rise in cytosolic free Ca^{++} concentration ([Ca]i) is presumed to play a key role in the release of hormones in stimulus-secretion coupling (10). [Ca]i is regulated by Ca^{++} influx and efflux across the plasma membrane, and the redistribution of intracellular Ca^{++}. Voltage-dependent Ca^{++} channels as well as receptor-operated Ca^{++} channels are considered to be main pathways of Ca^{++} influx (11).

Abbreviations; CT; calcitonin, [Ca]i; cytosolic free Ca concentration, quin-2/AM; quin-2 tetraacetoxymethyl ester, HEPES; 4 (-2 hydroxy ethyl)-1-piperazine ethane sulfonic acid, EGTA; ethylene glycol (bis (β-amino ethyl ether)-N,N,N',N'-tetraacetic acid, PBS; phosphate-buffered saline, DMEM; Dulbecco's modified Eagle's medium.

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Calcitonin (CT) is a calcium-lowering hormone, mainly produced by thyroidal C-cells in mammals. Its secretion is tightly regulated by extracellular Ca^{++} concentration both in vivo and in vitro (12). However, whether CT secretion is regulated by [Ca]i and related to gating of voltage-dependent Ca^{++} channels has not been decided.

Therefore we studied the effect of Bay K 8644, a putative Ca^{++} agonist, on CT secretion and on [Ca]i measured by a fluorescent Ca^{++} indicator, quin-2, in rMTC 6-23 cell line derived from a transplantable rat medullary thyroid carcinoma (13, 14).

Materials and methods:

rMTC 6-23 cells (American Type Culture Collection) were grown in monolayers in DMEM supplemented with 15% horse serum (Gibco) in a humidified atmosphere with 5% CO_2-95% air. Culture media were purchased from Flow Laboratories Inc. U.S.A. Quin-2/AM was from Dojin Chemical Laboratory, Japan. Bay K 8644 was a generous gift from Prof. Hoffmeister and Dr. Garthoff, Bayer AG, FRG. Nifedipine was provided by Bayer Yakuhin Ltd, Japan.

Calcitonin was measured by radioimmunoassay using anti-human CT antibody (15) which completely cross-reacted with rat CT.

Secretion experiment; After a 15 min preincubation in serum-free Ham's F12 medium (Ca^{++} 1.0mM, HEPES 20mM), confluent cells on replicate 35mm dishes were rinsed twice with PBS, and incubated with the fresh medium containing test agents (Bay K 8644, nifedipine) or vehicle alone. After an indicated time interval, the medium was collected, centrifuged, and kept at -20C until assayed. All these procedures were performed under dim light due to the photolability of the dihydropyridines. Dihydropyridines were dissolved in ethanol. CT concentration was corrected by cell protein, measured by the method of Lowry et al (16).

[Ca]i measurement; [Ca]i was measured by quin-2. Cells dispersed by 0.025% trypsin and 0.02% EDTA were kept in the serum-containing DMEM for 3 hr. Then $5X10^7$ cells were incubated with 50 μM quin-2/AM in the Ham's F12 medium for 20 min, followed by another 50 min incubation with 10 volumes of the medium. After the incubation period, aliquots of $5X10^6$ cells were washed, centrifuged, and resuspended in 2ml of HEPES-buffered saline (NaCl 145mM, Na_2HPO_4 1mM, $CaCl_2$ 1mM, $MgSO_4$ 0.5mM, HEPES 10mM, pH 7.4 at 37C). Fluorescence from the cells was monitored by a Hitachi spectrofluorimeter 650-40 (excitation 339nm, emission 492nm, slit width 4nm, 10nm, respectively) with or without test agents. Calibration of the fluorescence (F) was performed by lysing the cells with 50 μM digitonin (Fmax), chelating Ca^{++} with EGTA and Tris base (Fmin), as described by Tsien et al (17). [Ca]i was calculated from the equation;

$$[Ca]i = Kd\ (F-Fmin)/(Fmax-F),\quad Kd = 115nM$$

Data were presented as the mean $\pm$ S.E.M. Statistical significance was assessed by Student's t-test.

Results:

The time course of CT release with or without Bay K 8644 is demonstrated in Fig.1. The secretion rate with 10^{-6} M Bay K 8644 was greatest during the

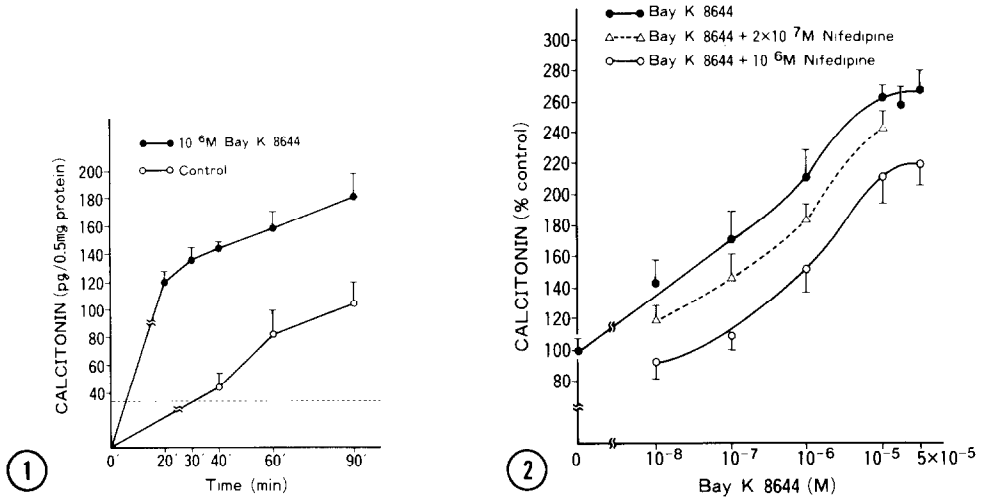

Fig. 1. Time course effect of Bay K 8644 on CT release. Bay K 8644 at the concentration of 10^{-6} M showed the maximal secretion rate during the first 20 min. Dotted line (----) shows the detectable level with the assay. Each point gives the mean ± S.E.M. from six dishes.

Fig. 2. Dose-response curve to Bay K 8644 (●—●)on CT secretion, and that with nifedipine of $2X10^{-7}$ M (Δ--Δ) or 10^{-6} M (○—○). CT secreted into the medium for 60 min was measured. Nifedipine caused the shift of the curve to Bay K 8644 alone to the right.

first 20 min, whereas that of the control was almost linear within 90 min. At 60 min, Bay K 8644 caused a two-fold secretion over the control. In the absence of Bay K 8644 , CT levels were undetectable within the first 30 min.

Dose-response effect of Bay K8644 on CT secretion is shown in Fig.2. Incubation time was set at 60 min, because the basal CT concentration was scarcely detectable at 40 min. Bay K 8644 caused a significant increase of CT secretion at the concentration of 10^{-8} M (146±14.0% of control, n=6, $p<0.02$), and showed a dose-dependent stimulation up to 10^{-5} M, where the secretion reached the plateau (267±9.3% of control, n=6, $p<0.01$). The effect of nifedipine in the presence of Bay K 8644 was also examined. An addition of $2X10^{-7}$M nifedipine induced a parallel right shift of the respnse curve to Bay K 8644, and 10^{-6} M nifedipine caused a further shift of the curve, indicating a competitive antagonism between these drugs.

The effect of Bay K 8644 on CT secretion in the absence of extracellular $Ca^{++}$was tested, by adding EGTA into the medium. In the presence

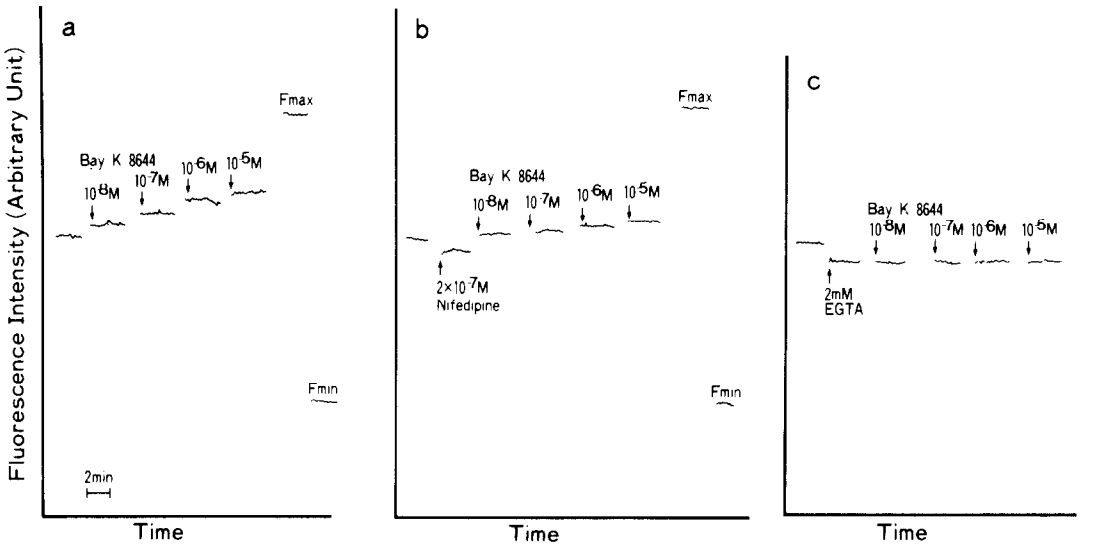

Fig. 3. Typical tracings of the monitoring the fluorescence from quin-2 loaded cells. Cells in the HEPES-buffered saline were stirred continuously with a magnetic stirrer.

3-a. Bay K 8644 was added into the "saline" stepwisely. A dose-dependent increase in [Ca]i was demonstrated.

3-b. An addition of $2X10^{-7}$ M nifedipine caused a fall in [Ca]i from the basal level. This effect was antagonized by Bay K 8644.

3-c. In the presence of EGTA in the "saline", the effect of Bay K 8644 was abolished.

of 2mM EGTA in the medium, Bay K 8644 at the concentration of 10^{-6} M failed to stimulate CT secretion above the detectable level (<25pg/ml/60 min/dish).

The representative records of monitoring of the fluorescence from the quin-2 loaded cells are illustrated in Fig.3. Bay K 8644 induced an immediate and dose-dependent rise in [Ca]i (Fig. 3-a). An addition of $2X10^{-7}$ M nifedipine caused a fall in [Ca]i (134±4.8 vs 112±3.0 nM, $p < 0.01$) which was elevated above the basal level with 10^{-8} M Bay K 8644 (Fig. 3-b). [Ca]i with Bay K 8644 at the concentration of 10^{-8}M, 10^{-7} M, and 10^{-6} M was 192±7.0, 231 ±6.4, and 330±10.4 nM, whereas those values after the addition of $2X10^{-7}$ M nifedipine were 148±3.8, 201±7.4, and 236±5.8 nM, respectively (Fig.4).

No rise in [Ca]i was observed with any concentration of Bay K 8644 in the presence of 2mM EGTA in the buffer (Fig. 3-c).

Discussions:

CT secretion is known to be influenced by various ions, particularly Ca^{++} (12, 14), but its relationship with [Ca]i or with the transmembrane Ca^{++}

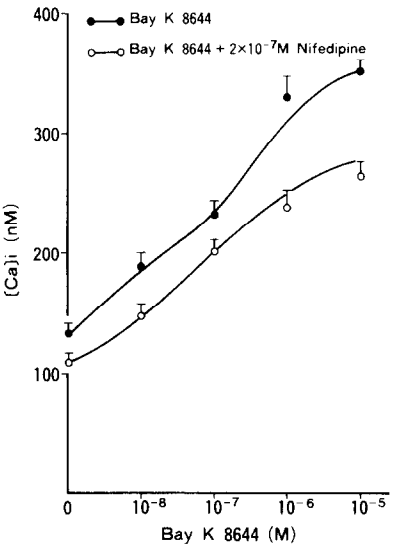

Fig. 4. A rise in [Ca]i induced by Bay K 8644 (10^{-8} M - 10^{-5} M) (●—●), and inhibition by $2X10^{-7}$ M nifedipine (○—○).

influx has not yet been clarified. The present data demonstrated a definite enhancement of Ca^{++}influx by Bay K 8644 with a subsequent rise in [Ca]i and a stimulation of CT secretion in the rat C-cell line. In view of the dependence on the presence of extracellular Ca^{++} and the competitive antagonism with nifedipine, Bay K 8644 seems most likely to act at the voltage-dependent Ca^{++} channel. Although Ca^{++} flux studies have shown that Bay K 8644 stimulates ^{45}Ca uptake (2, 6, 7, 9), this is the first report with a direct evidence that this compound raises [Ca]i in the hormone-secreting cell.

The influence on cellular functions by trypsinization and/or quin-2 loading should be minimal, if present, as judged by the viability of the cells (trypan blue dye exclusion >90%) and the hormonal response to secretagogues, such as high extracellular Ca^{++} or K^{+} (data not shown). The range of the effective concentration of this drug in rMTC 6-23 cell was similar to that in adrenal chromaffin cell (7), and pituitary cell line (8). In the heart,however, the positive inotropic effect was reported to be maximal at the concentration of 10^{-7} M (2). This discrepancy may be partly due to the difference of the susceptibility to dihydropyridines among the tissues or cells.

In summary, there seems to exist a regulatory site in or near the voltage-dependent Ca^{++} channel in CT producing cells, and Ca^{++} current through the channel could participate in the regulation of [Ca]i and CT secretion, suggesting a physiological role of Ca^{++} channel in the hormone secretion. Further investigations will be required to elucidate the mechanism of Ca^{++} action as an intracellular messenger.

Bay K 8644 appears to be a useful tool for not only exploring the property of the Ca^{++} channels but also manipulating intracellular Ca^{++}.

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