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Influence of hypothyroid state on $^{45}Ca^{2+}$ influx and sensitivity of rat uterus to nifedipine and diltiazem

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Abstract

The influence of methimazole-induced hypothyroidism on spontaneous rhythmic contractions and Ca^{2+} channel function of rat uterus was examined. Hypothyroidism significantly reduced the amplitude and frequency of spontaneous rhythmic contractions. Nifedipine (10^{-12}–10^{-6} M) and diltiazem (10^{-9}–10^{-4} M) caused concentration-related inhibition of the myogenic responses of the oestrogenised rat uterus obtained from both eu- and hypothyroid rats. However, nifedipine was less potent (IC_{50}; 5.4×10^{-9} M; $n = 6$) in hypothyroid rat uterus as compared to euthyroid controls (IC_{50}: 8.13×10^{-12} M; $n = 9$) to inhibit the rhythmic contractions. Similarly, diltiazem was less potent (IC_{50}: 4.57×10^{-6} M; $n = 9$) to inhibit the uterine spontaneous contractions in hypothyroid than in euthyroid rat uterus (IC_{50}: 6.4×10^{-8} M; $n = 6$). A similar decrease in the sensitivity to nifedipine and diltiazem for reversal of K^+ (100 mM)-induced tonic contraction was observed in uterus obtained from hypothyroid rats compared to the controls. Both nifedipine and diltiazem were less potent for causing concentration-related inhibition of K^+-stimulated $^{45}Ca^{2+}$ influx in uterine strips taken from the hypothyroid rats. Thus, the IC_{50} values of nifedipine (1.83×10^{-8} M; $n = 12$) and diltiazem (1.8×10^{-6} M; $n = 9$) were significantly greater in tissues obtained from hypothyroid rats compared to the controls (IC_{50} of nifedipine, 1.15×10^{-11} M; $n = 12$, diltiazem, 8.1×10^{-8} M; $n = 8$). Nifedipine-sensitive influx of $^{45}Ca^{2+}$- stimulated either by K^+ (100 mM) or Bay k8644 (1,4-dihydro-2,6-dimethyl-5-nitro-4-[2′-(trifluromethyl)phenyl]-3-pyridine carboxylic acid methyl ester) (10^{-8} M) was significantly less in uterine strips from hypothyroid rats compared to the controls. The results of the present study suggest that the inhibition of uterine rhythmic contractions may be attributable to a reduction in rat myometrial Ca^{2+} channel function in the hypothyroid state.

Keywords: Uterus; Hypothyroidism; Ca^{2+} channel; Nifedipine; Diltiazem

1. Introduction

Reproductive dysfunctions, ranging from abnormal sexual development to infertility and irregularities in reproductive cycle, have long been associated with thyroid disorders (Thomas and Reid, 1987). Hypothyroidism has also been reported to influence uterine morphology. For instance, in rats made hypothyroid with methimazole, there is a reduction in absolute volume of endometrium as well as a decrease in muscle layer (Inuwa and Williams, 1996). Although there is evidence for the presence of thyroid hormone receptors in uterine nuclei and of a very significant influence of thyroid hormones on the contractility of myometrium (Evans et al., 1983; Mukku et al., 1984; Medieros and Calixto, 1989), there is little information on the influence of thyroid status on the cellular basis of altered contractility, especially with respect to Ca^{2+} handling by the tissue. There are a number of reports, however, on the effect of thyroid status on calcium channel function in the cardiovascular system. For instance, ventricular myocytes obtained from hypothyroid guinea pigs showed a reduced peak Ca^{2+} channel current (Binah et al., 1987). It has also been shown that hypothyroidism induces a moderately lower (reduced by about 23%) myocardial density of 1,4-dihydropyridine receptors in rats (Wibo et

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al., 1995). Hyperthyroidism, on the other hand, increased the calcium channel function in chick ventricular myocytes and human atrial myocytes. Chick ventricular cells grown in triiodothyronine (T_3) showed a marked increase in dihydropyridine binding as well as an augmented transsarcolemmal Ca^{2+} influx (Kim et al., 1987). The increased calcium channel function was also evident in patients with latent hypothyroidism (a condition in which there is a suppressed thyroid stimulating hormone but normal circulatory thyroid hormones) wherein there was an increased expression and activity of L-type Ca^{2+} channels (Kreuzberg et al., 2000).

Ca^{2+} entry through voltage-dependent Ca^{2+} channels plays a critical role in myometrial contractility (Wray, 1993). These channels are subject to hormonal regulation as evident from an increase in channel density with corresponding increase in $^{45}Ca^{2+}$ influx in the myometrium of estrogen-treated rat uterus (Batra, 1987). In vitro application of β-estradiol has been reported to inhibit calcium channel currents in pregnant rat myometrial cells (Yamamoto, 1995; Okabe et al., 1999). In view of the lack of information concerning the role of thyroid hormones in affecting Ca^{2+} channel function in the myometrium, the aim of the present study was to examine the influence of methimazole-induced hypothyroidism on the depolarization-induced $^{45}Ca^{2+}$ influx and on the sensitivity of spontaneously contracting rat uterus to calcium channel blockers such as, nifedipine and diltiazem. There is also no information on the influence of hypothyroidism on the spontaneous rhythmic contractions of rat uterus. It was, therefore, of interest to examine the link between calcium channel function and mechanical responses as influenced by the thyroid status.

2. Materials and methods

2.1. Induction of hypothyroidism

Adult female Sprague–Dawley rats (180–200 g), obtained from the Central Drug Research Institute, Lucknow, were used in the present study, as per the Institutional Policy on Animal Use. A hypothyroid status was induced by 15-day treatment of rats with methimazole (10 mg/kg; i.p.; Swan, 1989). Control rats (euthyroid) received a comparable volume of normal saline i.p. The thyroid status in control and methimazole-treated rats was evaluated by measuring plasma thyroxine (T_4) and triiodothyronine (T_3) using a radioimmunoassay.

2.2. Tension experiments

Rats were treated with diethylstilboesterol (1.5 mg/kg) for two consecutive days before they were killed by cervical dislocation. The uterine horns were isolated and placed in cold oxygenated Ringer-Locke solution of the following composition (mM): NaCl, 136.9; KCl, 5.6; $NaHCO_3$,11.9; $CaCl_2 \cdot 2H_2O$, 2.2 and glucose, 5.6. The longitudinal strips, approximately 3×10 mm from each mid-horn region, were dissected out and were suspended in an organ bath containing 20 ml Ringer-Locke modified solution, bubbled continuously with O_2 at 37 ± 0.5°C under a resting tension of 1 g and equilibrated for a period of 1 h. Isometric contractions were recorded by a force transducer connected to an ink-writing oscillograph (Recorders and Medicare, India).

2.3. $^{45}Ca^{2+}$ influx measurement

The net increase in the unidirectional entry of Ca^{2+} into the uterine smooth muscle was estimated by measuring the increase in $^{45}Ca^{2+}$ content of uterine strips obtained from eu- and hypothyroid rats as per the method of Batra (1987). In brief, uterine strips weighing about 6–8 mg were equilibrated for 45 min in physiological Na-HEPES solution of the following composition (mM): NaCl, 135; KCl, 4.6; $MgCl_2$, 1.2; $CaCl_2$, 1.5; glucose, 11 and HEPES, 10; pH 7.4, maintained at 37°C and bubbled with O_2. The tissues were incubated for 15 min with different concentrations of calcium channel blockers such as nifedipine (10^{-11}–10^{-5} M) and diltiazem (10^{-9}–10^{-5} M) before the strips were equilibrated for an additional 2 min with Na-HEPES solution containing $^{45}Ca^{2+}$ (1–1.5 μCi/ml) to allow the exchange of extracellular Ca^{2+} with the tracer. For stimulation of $^{45}Ca^{2+}$ influx into the strips, the tissues were exposed for 2 min to a $^{45}Ca^{2+}$-containing K^+-depolarising solution in which NaCl was replaced by 100 mM KCl. Unstimulated influx was measured in the tissues incubated in Na-HEPES solution. Uterine strips were then washed for 45 min in 5 ml ice-cold Ca^{2+}-free EGTA-HEPES solution with vigorous bubbling with O_2 to remove the loosely bound extracellular tracer. After washing with EGTA solution, the tissues were lightly blotted with filter paper, placed in scintillation vials and weighed. The tissues were digested using the method of Pfeffer et al. (1971). The radioactivity of the tissues was measured using a liquid scintillation counter (LKB, USA).

2.4. Drugs

Nifedipine and methimazole were purchased from Sigma (USA). Diltiazem was a gift from Marion Laboratory (USA). 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2′-(trifluromethyl)phenyl]-3-pyridine carboxylic acid methyl ester (Bay K8644) was a gift from Bayers (Germany).

2.5. Statistics

Results are given as means ± S.E.M. Student's unpaired *t*-test was employed to test for significance at the level of

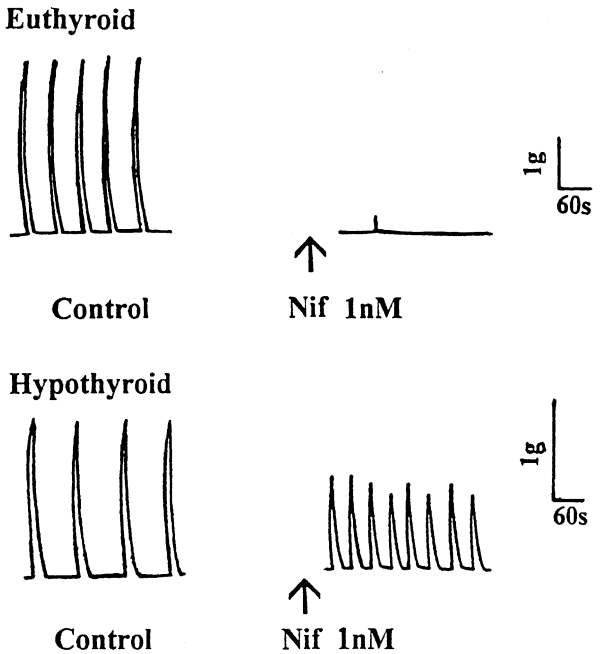

Fig. 1. Influence of thyroid status on the spontaneous rhythmic contractions of oestrogenised rat uterus and the effect of nifedipine (1 nM) on these contractions.

$P < 0.05$. IC_{50} values were calculated by regression analysis (Snedecor and Cochran, 1980).

3. Results

3.1. Effects of methimazole on plasma T_3 and T_4 levels

In the rats treated with methimazole for 15 days, a significant ($P < 0.01$) decrease in plasma T_3 level (0.46 ± 0.05 ng/ml; $n = 6$) was observed as compared to that in the saline-treated euthyroid controls (1.06 ± 0.08 ng/ml; $n = 6$). Similarly, a significant reduction in T_4 level (26.5 ± 1.52 ng/ml; $n = 6$) occurred in the methimazole group compared to the saline-treated controls (52.24 ± 1.96 ng/ml; $n = 6$).

3.2. Effect of thyroid status on the spontaneous rhythmic contractions of rat uterus

The influence of thyroid status on spontaneous rhythmic contractions of rat uterus is shown in Fig. 1. The amplitude of rhythmic contractions in euthyroid rat uterus was 2.7 ± 0.1 g ($n = 23$) which was significantly ($P < 0.05$) greater than that for the hypothyroid status (1.51 ± 0.05 g; $n = 23$). Similarly, a decrease in the frequency of spontaneous contractions was noted in hypothyroid rat uterus (0.77 ± 0.03/min; $n = 23$) compared to the euthyroid controls (0.92 ± 0.02/min; $n = 23$). Methimazole had no effect on the wet weight of uterine horns taken from oestrogenised rats. The tissue weight of virgin rats treated with estradiol alone was 0.40 ± 0.04 g ($n = 6$) compared to that for rats treated with methimazole and estradiol (0.53 ± 0.07 g; $n = 6$).

3.3. Effect of thyroid status on the sensitivity of rat uterus to nifedipine and diltiazem

The inhibitory concentration–response curves for nifedipine and diltiazem are shown in Fig. 2. The amplitude of control rhythmic contractions in uterus from eu- and hypothyroid rats was 2.1 ± 0.1 g ($n = 9$) and 1.4 ± 0.1 g ($n = 6$), respectively. Nifedipine (10^{-12}–10^{-6} M), added cumulatively at intervals of 1 log unit produced a concentration-related reduction in the amplitude of rhythmic contractions of uterine muscle taken from eu- and hypothyroid rats. Rhythmic contractions were abolished by nifedipine 1×10^{-9} M in euthyroid condition whereas a much higher

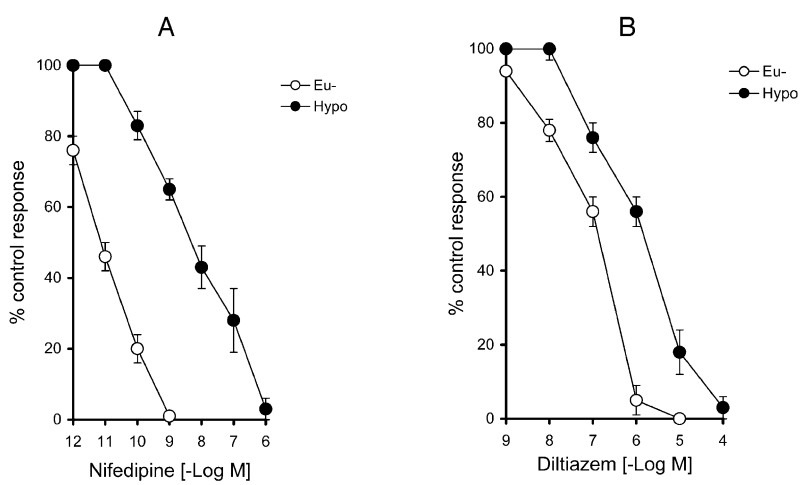

Fig. 2. Effect of thyroid status on the sensitivity of the amplitude of spontaneous rhythmic contractions of rat uterus to nifedipine and diltiazem. Fig. 2A depicts the concentration-related reduction of the amplitude of rhythmic contractions by nifedipine (10^{-12}–10^{-6} M) added cumulatively at an interval of one log unit. (B) Concentration-related inhibition of the rhythmic contractions by diltiazem (10^{-9}–10^{-4} M).

Table 1
Effect of nifedipine and diltiazem on the amplitude of rhythmic contractions, K^+-induced tonic contraction and K^+-stimulated net $^{45}Ca^{2+}$ influx in oestrogenised rat uterus obtained from eu- and hypothyroid rats

Thyroid state	Nifedipine (IC_{50})	Diltiazem (IC_{50})
1. Rhythmic contractions:		
Euthyroid	8.13×10^{-12} M	6.40×10^{-8} M
	C.L.5×10^{-12}–1.60×10^{-11} M	C.L.4.1×10^{-8}–1.02×10^{-7} M
	($n = 9$)	($n = 6$)
Hypothyroid	5.35×10^{-9} M	4.57×10^{-6} M
	C.L.1.33×10^{-9}–2.15×10^{-8} M	C.L. 9.74×10^{-7}–2.14×10^{-5} M
	($n = 6$)	($n = 9$)
2. K^+-induced tonic contraction		
Euthyroid	4.37×10^{-12} M	9.79×10^{-8} M
	C.L. 2.99×10^{-12}–6.38×10^{-12} M	C.L. 6.25×10^{-8}–1.53×10^{-7} M
	($n = 6$)	($n = 6$)
Hypothyroid	8.15×10^{-9} M	2.5×10^{-6} M
	C.L. 5.18×10^{-9}–1.28×10^{-8} M	C.L. 7.13×10^{-7}–8.85×10^{-6} M
	($n = 5$)	($n = 6$)
3. K^+-stimulated net $^{45}Ca^{2+}$ influx		
Euthyroid	1.14×10^{-11} M	8.10×10^{-8} M
	C.L. 5.6×10^{-12}–2.34×10^{-11} M	C.L. 5.73×10^{-8}–1.20×10^{-7} M
	($n = 12$)	($n = 8$)
Hypothyroid	1.83×10^{-8} M	1.80×10^{-6} M
	C.L. 1.15×10^{-8}–2.9×10^{-8} M	C.L. 9.94×10^{-7}–3.39×10^{-6} M
	($n = 12$)	($n = 9$)

C.L.-95% confidence limits; *n*-Number of observations.

concentration of nifedipine (1×10^{-6} M) was required to abolish the rhythmic contractions in the uterus obtained from hypothyroid rats. The reduced sensitivity of spontaneous rhythmic contractions to nifedipine in hypothyroid rat uterus is evident from the increase in the IC_{50} value compared to the controls as shown in Table 1. In the diltiazem group, the amplitude of spontaneous rhythmic contractions was 2.67 ± 0.2 g ($n = 6$) and 1.6 ± 0.12 g ($n = 9$) in eu- and hypothyroid rat uterus, respectively. As observed with nifedipine, a marked decrease in the sensitivity of the myogenic contractions to diltiazem was evident in the hypothyroid rat uterus. The spontaneous contractions were abolished at 1×10^{-5} and 1×10^{-4} M, respectively, in eu- and hypothyroid conditions. The IC_{50} of diltiazem to inhibit the amplitude of spontaneous contractions is presented in Table 1.

3.4. Influence of the thyroid status on the reversal of K^+-induced tonic contractions by nifedipine and diltiazem

In isolated rat uterus, K^+ (100 mM) depolarising solution induced a biphasic contractile response consisting of a

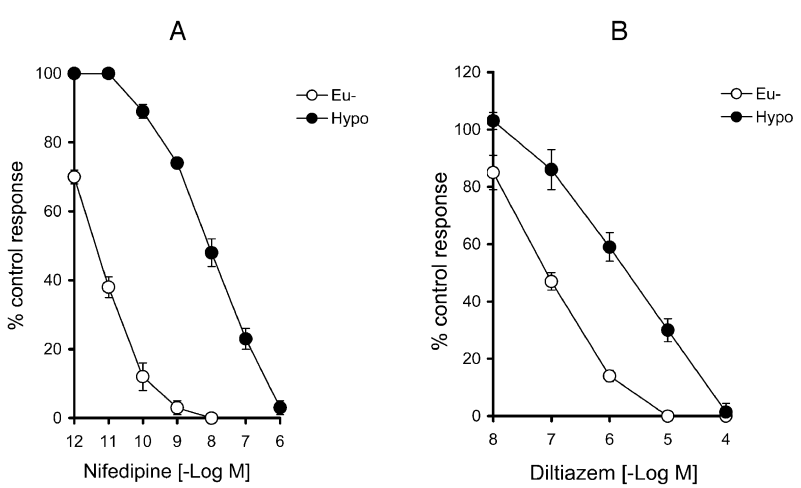

Fig. 3. Effect of thyroid status on the concentration-related relaxation of the tonic component of K^+ (100 mM)-contracted isolated rat uterus by nifedipine (A) and diltiazem (B).

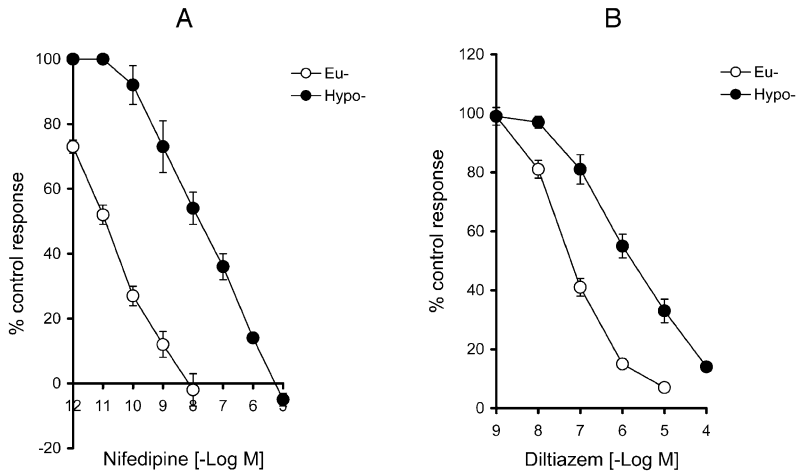

Fig. 4. Effect of thyroid status on the concentration-related inhibition of K^+ (100 mM)-stimulated net $^{45}Ca^{2+}$-influx by nifedipine (A) and diltiazem (B) in rat uterine strips.

fast phasic component followed by a slow sustained contraction which stabilized in about 5 min. The reversal of tonic contraction by Ca^{2+} channel blockers was evaluated to determine the sensitivity of the Ca^{2+} channel ligands in eu- and hypothyroid rat uterus. The amplitude of the tonic component of K^+-induced contraction was 1.38 ± 0.08 g ($n = 6$) and 1.14 ± 0.05 g ($n = 5$), respectively, in eu- and hypothyroid rat uterus. Nifedipine (10^{-12}–10^{-5} M), added cumulatively, caused concentration-related relaxation of the K^+-contracted uterine smooth muscle taken from both eu- and hypothyroid rats (Fig. 3). Complete reversal of the tonic contraction occurred at 1×10^{-8} M of nifedipine for both. The tissues obtained from hypothyroid rats, however, had a decreased sensitivity to nifedipine which is evident from the increase in its IC_{50} value compared to controls (Table 1). In the diltiazem group, the absolute tension of K^+-induced control tonic contraction in eu- and hypothyroid tissues was 1.56 ± 0.06 g ($n = 6$) and 1.0 ± 0.08 g ($n = 5$), respectively. The concentration of diltiazem required to completely reverse the tonic contractions was 1×10^{-5} and 1×10^{-4} M, respectively, in eu- and hypothyroid rat uterus. A marked decrease in the sensitivity to relaxation by diltiazem of K^+-induced tonic contractions was also observed in the uterine smooth muscle taken from hypothyroid rats with a resultant increase in its IC_{50} value compared to euhyroid controls (Table 1).

3.5. Effect of thyroid status on the sensitivity of K^+-stimulated $^{45}Ca^{2+}$ influx to nifedipine and diltiazem

The net influx of $^{45}Ca^{2+}$ stimulated by 100 mM K^+ was 0.096 ± 0.007 μmol ($n = 12$) and 0.0647 ± 0.006 μmol/g tissue ($n = 12$) in uterine strips obtained from eu- and hypothyroid rats, respectively. Nifedipine (10^{-12}–10^{-6} M) and diltiazem (10^{-9}–10^{-5} M) inhibited the K^+ (100 mM)-stimulated net $^{45}Ca^{2+}$ influx in a concentration-related manner (Fig. 4). As shown in the figure, the concentration–response curves of nifedipine and diltiazem were shifted to the right in hypothyroid rat uterus as compared to the controls. The IC_{50} values of nifedipine are presented

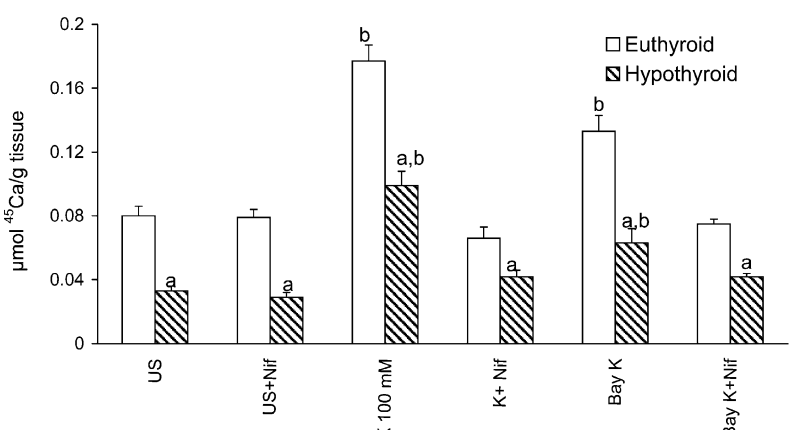

Fig. 5. Effect of thyroid status on basal, K^+ (100 mM) and Bay k8644 (10^{-8} M)-stimulated $^{45}Ca^{2+}$ influx in the presence and in the absence of nifedipine (10^{-6} M). (a) $P < 0.05$ as compared to euthyroid controls. (b) $P < 0.05$ as compared to unstimulated influx.

in Table 1. Similarly, the IC_{50} of diltiazem for inhibiting $^{45}Ca^{2+}$ influx into uterine strips from hypothyroid rats was significantly greater than in the controls (Table 1).

3.6. Effect of thyroid status on nifedipine-sensitive $^{45}Ca^{2+}$ influx in uterine strips stimulated with high K^+ (100 mM) and Bay k8644

The influence of thyroid status on K^+- and Bay k8644-stimulated $^{45}Ca^{2+}$ influx in rat uterine strips is shown in Fig. 5. The unstimulated influx of $^{45}Ca^{2+}$, which was not sensitive to block by nifedipine (10^{-6} M), was significantly ($P < 0.05$) higher in the controls (0.08 ± 0.006 μmol/g tissue; $n = 12$) than in the hypothyroid rat uterus (0.033 ± 0.003 μmol/g tissue; $n = 12$). A 2-min stimulation with K^+ (100 mM) markedly increased the $^{45}Ca^{2+}$ influx into the uterine strips obtained from both eu- and hypothyroid rats. In terms of absolute values, the $^{45}Ca^{2+}$ influx in response to K^+-depolarising solution was greater in euthyroid controls than in the hypothyroid ones. Similarly, Bay k8644-stimulated $^{45}Ca^{2+}$ influx in control uterine strips was significantly greater than in the tissues obtained from hypothyroid rats. Pretreatment of the tissues for 15 min with nifedipine (10^{-6} M) blocked the $^{45}Ca^{2+}$-influx stimulated by either K^+ (100 mM) or Bay k8644 (10^{-6} M) in tissues taken from both eu- and hypothyroid rats.

4. Discussion

The three most important observations of the present study are that (i) the spontaneous rhythmic contractions (both amplitude and frequency) are reduced; (ii) the sensitivity to L-type Ca^{2+} channel blockers, nifedipine and diltiazem, is markedly reduced; and (iii) $^{45}Ca^{2+}$ influx stimulated by K^+ depolarization and Bay k8644 is significantly less in uterine tissues obtained from hypothyroid rats than from the euthyroid controls. Hypothyroidism in response to methimazole treatment was confirmed by a significant decrease in circulatory T_3 and T_4 levels. The reduction in the amplitude and frequency of rhythmic contractions could not be attributed to a change in uterine muscle mass as there was no significant change in wet weight of uterine horns in methimazole-treated rats compared to the euthyroid controls. The reduced Ca^{2+} channel function may have been responsible for a reduction in the amplitude of rhythmic contractions in hypothyroid uterus. It is well established that both rhythmic contractions and contractions caused by K^+ depolarization in uterine smooth muscle are critically dependent on extracellular Ca^{2+} (Wray, 1993) and L-type Ca^{2+} channel blockers are potent inhibitors of these contractions (Hollingsworth and Downing, 1988). In an earlier study from our laboratory, we have shown that hypothyroidism markedly depressed the spontaneous rhythmic contractions in rat portal vein and this was attributed to an increase in the opening of ATP-sensitive K^+ channels (Jagadish et al., 1996). Whether uterine K^+ channels are affected by hypothyroidism is not known. But hypothyroidism has been reported to decrease the expression of voltage-dependent K^+ (K_v) channel mRNAs in rat myocardium (Nishiyama et al., 1998; Ojamma et al., 1999). It is possible that the alteration in the mechanical responses of uterus in hypothyroidism results from a change in both Ca^{2+} and K^+ channel function. However, we have restricted our studies to uterine Ca^{2+} channel function as influenced by hypothyroidism.

It was observed that nifedipine and diltiazem, the prototype Ca^{2+} channel blockers, caused concentration-related inhibition of spontaneous rhythmic contractions in the oestrogenised uterus taken from both eu- and hypothyroid rats. As reported in the literature (Hollingsworth and Downing, 1988), nifedipine was more potent than diltiazem to inhibit the rhythmic contractions. A rightward shift in the concentration–response curves of nifedipine and diltiazem in hypothyroid tissues indicates that Ca^{2+} channel blockers are less potent to inhibit the uterine contractions in the presence of thyroid deficiency. It is known that the membrane potential is a critical determinant of the potency of Ca^{2+} channel blockers to block L-type Ca^{2+} channels (Sanguinetti and Kass, 1984). In order to rule out a possible influence of membrane potential affecting the potency of nifedipine and diltiazem, we conducted some experiments in high-K^+ solution that caused a biphasic contraction of the uterus taken from both eu- and hypothyroid rats. It was observed that both nifedipine and diltiazem were less potent to reverse the tonic component of K^+ contraction in uterine strips obtained from hypothyroid rats than those from euthyroid ones. The observations from the contraction studies were further confirmed by the findings that concentration–response curves of nifedipine and diltiazem for blocking K^+-stimulated $^{45}Ca^{2+}$ influx in uterine strips obtained from hypothyroid rats were shifted rightward as compared to the euthyroid controls. These observations suggest that hypothyroidism reduces the affinity of L-type Ca^{2+} channels to the Ca^{2+} channel blockers. Although there are no reports regarding the regulation of Ca^{2+} channel function in myometrium by thyroid hormones, sex hormones such as estrogen have been reported to increase the affinity of L-type Ca^{2+} channels in rat uterus (Ishi et al., 1986).

Consistent with the reduced sensitivity of L-type calcium channels to nifedipine and diltiazem, we observed that hypothyroidism markedly inhibited nifedipine-sensitive $^{45}Ca^{2+}$ influx, stimulated by either high K^+ or the Ca^{2+} channel agonist, Bay k8644, in uterine strips taken from hypothyroid rats when compared to the controls. Although there is no information on the effect of thyroid status on Ca^{2+} channel function of uterine smooth muscle, there are several reports on the influence of thyroid status on the L-type Ca^{2+} channel function in the myocardium. For instance, hypothyroidism was reported to inhibit the

peak L-type Ca^{2+} channel current in guinea pig ventricular myocytes (Binah et al., 1987). Further, in a ligand binding study, hypothyroidism was shown to lower the density of myocardial 1,4-dihydropyridine receptors in rats (Wibo et al., 1995). Hyperthyroidism, on the other hand, has been reported to increase cardiac Ca^{2+} channel function (Kim et al., 1987; Kreuzberg et al., 2000). In view of the presence of nuclear thyroid hormone receptors in the rat uterus (Evans et al., 1983), it is possible that thyroid hormones regulate uterine Ca^{2+} channels in a manner similar to their influence on cardiac Ca^{2+} channels.

In conclusion, the present study on the rat uterus provides evidence for the first time that hypothyroidism reduces the spontaneous rhythmic contractions, and the influx of Ca^{2+} through voltage-dependent L-type Ca^{2+} channels, as well as decreases the sensitivity of the uterine smooth muscle to L-type Ca^{2+} channel blockers such as nifedipine and diltiazem. The decrease in L-type Ca^{2+} channel function, however, should be confirmed directly by further studies using electrophysiological methods to measure directly ion currents in different thyroid states.

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