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The effects of α-adrenoceptor agonists on intracellular Ca^{2+} levels in freshly dispersed single smooth muscle cells from rat tail artery

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1 The presence of functional α-adrenoceptors in freshly dispersed single smooth muscle cells from rat tail arteries was investigated by use of selective α-adrenoceptor agonists and antagonists.

2 Cirazoline, a selective α_1-adrenoceptor agonist, caused a prazosin-sensitive, rapid but transient increase in intracellular Ca^{2+}, which was partially inhibited by the voltage-dependent Ca^{2+} channel blocker, nifedipine.

3 TL99, an α_2-adrenoceptor agonist, in the presence of prazosin, initiated a slow and sustained increase in intracellular Ca^{2+} which was partially inhibited by yohimbine and almost completely blocked by nifedipine.

4 In rat tail artery, the effects (dose-response and time-response curves) of cirazoline and TL99 on intracellular Ca^{2+} levels in freshly dispersed single smooth muscle cells were comparable with those obtained with organ bath studies of ring preparations of artery.

5 In freshly dispersed single smooth muscle cells, the time-course response curves induced by the selective α_1-adrenoceptor agonist, phenylephrine and the selective α_2-adrenoceptor agonist, UK14304, were similar to those observed with cirazoline and TL99, respectively.

6 These results indicate that: (a) functional α_1- and α_2-adrenoceptors are present in freshly dispersed single smooth muscle cells from rat tail artery and (b) α_1- and α_2-adrenoceptors are coupled to different cellular processes that lead to an increase in intracellular Ca^{2+}.

Keywords: Intracellular Ca^{2+}; single smooth muscle cells; α-adrenoceptors; rat tail artery

Introduction

An extensive literature from *in vivo* studies supports a role for both postjunctional α_1- and α_2-adrenoceptors in mediating pressor responses to α-adrenoceptor agonists. In contrast, with *in vitro* techniques, only a few studies have demonstrated α_2-mediated contractile responses in arterial smooth muscle (see Timmermans & van Zwieten, 1982). Furthermore, although there are very few reports of α-mediated responses in freshly dispersed vascular smooth muscle cells or cell cultures, responses to peptides seem to be well preserved (Bukoski, 1990; Papageorgiou & Morgan, 1991). In the rat tail artery, *in vitro* studies and radioligand binding assays indicate the presence of both postjunctional α_1- and α_2-adrenoceptors (Abe *et al.*, 1987; Cheung & Triggle, 1988). The present study describes the effects of α-adrenoceptor agonists on intracellular Ca^{2+} levels in freshly dispersed single smooth muscle cells from rat tail artery. The major objectives of the present study were to determine (a): whether α_1- and α_2-adrenoceptors could be demonstrated in freshly dispersed vascular smooth muscle cells and (b): by use of fura-2 Ca^{2+}-measurement techniques, the cellular pathways for Ca^{2+} entry/mobilization utilized by α_1- and α_2-adrenoceptors.

Methods

Smooth muscle cell dispersion

Male Sprague Dawley rats (300–400 g) were stunned by a blow to the head and exsanguinated. The ventral tail artery was excised, cleaned and cut into segments of approximately 3–4 cm length. The technique for cell dispersion was essentially that described by Bolzon & Cheung (1989). Each segment was tied at both ends onto polythylene tubing (PE 50) and perfused with a physiological salt solution I (PSS I) (composition in mM: NaCl 137, KCl 5.4, KH_2PO_4 0.4, $NaHCO_3$ 4.2, NaH_2PO_4 0.4, glucose 5.6, HEPES 10.0 and pH was adjusted to 7.4 with 1 M NaOH) at a rate of 0.05 ml min^{-1} at 37°C. After a 90 min equilibration period, the perfusate was changed to one containing 0.13% papain (BDH INC., Canada) and 0.04% collagenase (Yakult Corp., Japan) and 4 mM dithiothreitol (Sigma, U.S.A.). After 45 min of enzyme perfusion, the segment was cut free and transferred to enzyme-free solution, a longitudinal incision was made, and the tissue was gently pipetted to release the smooth muscle cells. The smooth muscle cells were characteristically oblong in shape, and fura-2 signals were collected from only those cells that responded with a contractile response to the agonists (as described below).

Intracellular Ca^{2+} measurement

Intracellular Ca^{2+} levels were assessed with the fluorescent calcium indicator fura-2. The smooth muscle cells were incubated with 8 μM fura-2-acetoxy methylester at room temperature for 30 min and then transferred to a perfusion chamber, the bottom of which consisted of a glass coverslip. After the cells settled in the chamber, they were perfused with physiological salt solution II (PSS II) (+/− antagonists or blocker) for 5–10 min and then perfused with agonist-containing-PSS II. PSS II consisted of (mM); NaCl 120, KCl 5.0, NaH_2PO_4 1.0, $NaHCO_3$ 25.0, $CaCl_2$ 2.5, $MgSO_4$ 1.0, glucose 11.0, and HEPES 5.0. Changes in fluorescence intensity of fura-2 at excitation wavelengths of 340 and 380 nm

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were monitored in single cells at room temperature, with an SLM-Aminco 1000 fluorometer (SLM Instruments Inc., U.S.A.) interfaced with a Nikon Diaphot inverted microscope. After subtracting the background intensity at 340 and 380 nm, $[Ca^{2+}]_i$ was determined as described by Grynkiewicz *et al.* (1985) from the equation:

$$[Ca^{2+}]_i = K_d\{(R-R_{min})/(R_{max}-R)\}(Fo/Fs)$$

where R is the ratio of emission (510 nm) intensity at 340 and 380 nm excitation after background correction, K_d is the dissociation constant for fura-2 and Ca^{2+} (assumed to be 200 nM, Williams *et al.*, 1987). Fo and Fs are the fluorescence intensity at 380 nm excitation for free and calcium-saturated dye, respectively. Rmin and Fo were determined by exposure of the cells to Br-A-23187 (17 μM) in Ca^{2+}-free PSS II containing 4 mM EGTA, and R_{max} and Fs by exposure of the cells to Br-A-23187 (17 μM) in 10 mM Ca^{2+}-PSS II, respectively.

Organ bath studies

Ventral tail arteries were cut into rings of approximately 5–6 mm in length. Each ring was suspended between platinum hooks and mounted under 1.5 g of passive tension in 10 ml organ baths containing Krebs solution, maintained at room temperature and bubbled with 5% CO_2 in O_2 (pH 7.4). Tissues were allowed to equilibrate in this medium for 90 min, during which the bathing Krebs solution was routinely changed every 20 min. Fifteen minutes before preparation of dose-response or time-response curves, 10^{-6} M cocaine and 10^{-6} M propranolol were added to all tissue baths. Isometric tension was recorded with a force displacement transducer coupled to a Grass polygraph model 7D. The Krebs solution had the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 12.5 and glucose 11.1.

Cirazoline was a generous gift from Synthelabo, France; UK14304 (5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline), a gift from Pfizer, U.K.; prazosin hydrochloride from Pfizer,

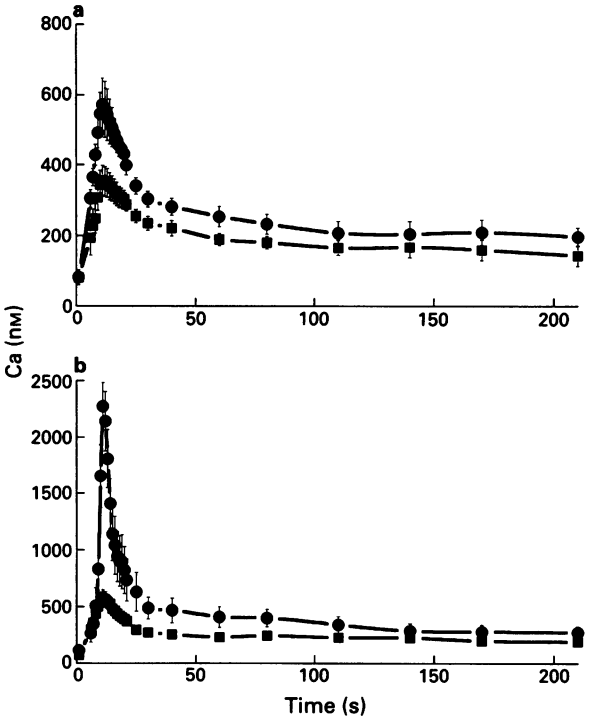

Figure 2 The effects of cirazoline on intracellular Ca^{2+} concentration in fura-2 loaded freshly dispersed single smooth muscle cells from rat tail artery. (a) Time course for cirazoline (0.1 μM) in the absence (●, $n = 6$) or presence of 0.1 μM prazosin (■, $n = 6$). (b) Time course for cirazoline (1 μM) in the absence (●, $n = 6$), or presence of 0.01 μM prazosin (■, $n = 6$). Each data point is plotted as mean ± s.e.mean.

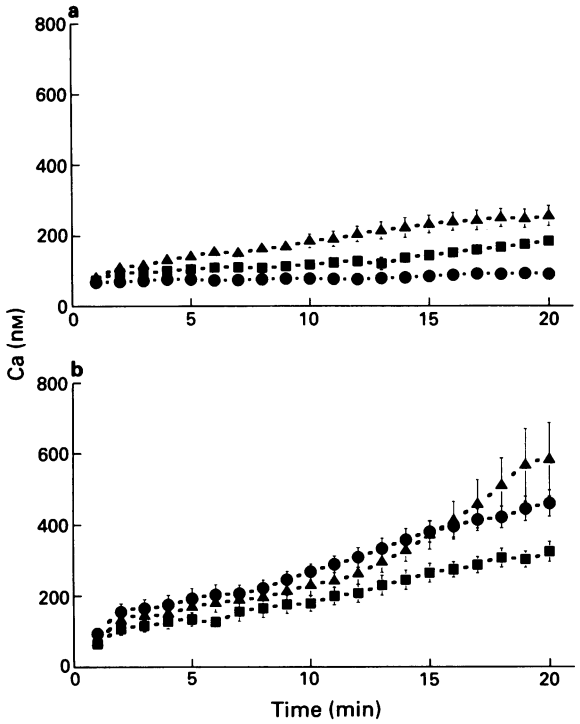

Figure 1 The effects of TL99 on intracellular Ca^{2+} concentrations in fura-2 loaded freshly dispersed single smooth muscle cells from rat tail artery. (a) Time course for 5 μM TL99 (+ 0.05 μM prazosin) in the absence (▲, $n = 6$) or presence of 1 μM yohimbine (■, $n = 8$) and control (perfusing the cells with PSS II) (●, $n = 4$). (b) Time course for 50 μM TL99 in the absence (▲, $n = 6$), and presence of 0.05 μM prazosin (●, $n = 6$), or 0.05 μM prazosin plus 1 μM yohimbine (■, $n = 6$). Every 60th data point is plotted as mean ± s.e.mean.

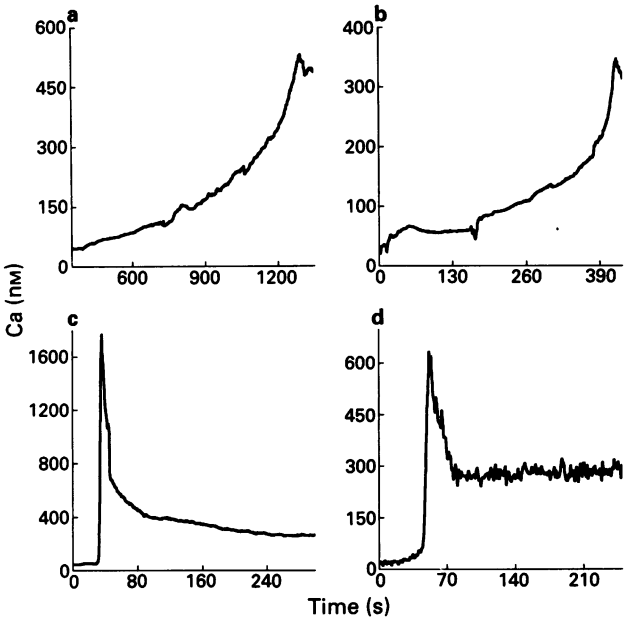

Figure 3 Original traces of intracellular Ca^{2+} content from fura-2-loaded freshly dispersed single smooth muscle cells from rat tail artery stimulated with (a) TL99 (50 μM), (b) UK14304 (10 μM), (c) cirazoline (1 μM) and (d) phenylephrine (1 μM).

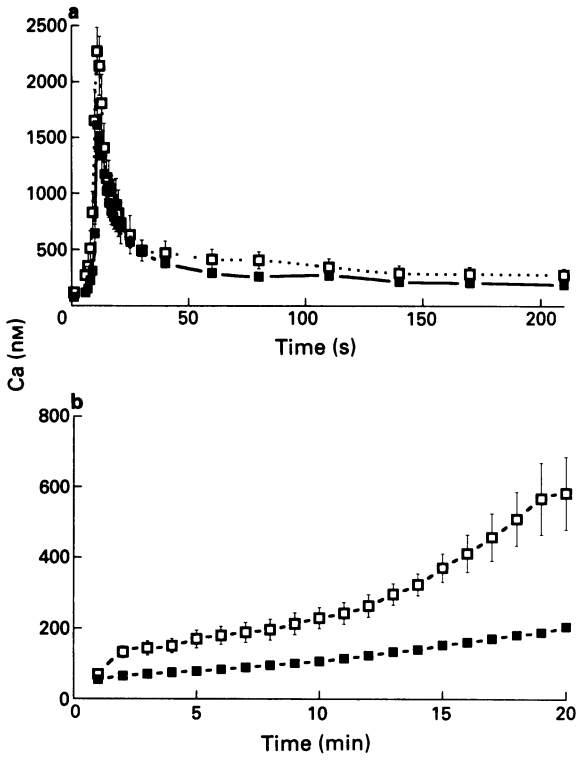

Figure 4 The effects of nifedipine on cirazoline and TL99-induced changes in intracellular Ca^{2+} levels in fura-2-loaded freshly dispersed single smooth muscle cells from rat tail artery. (a) Time course for cirazoline (1 μM) in the absence (□, $n = 6$), or presence of 0.1 μM nifedipine, (■, $n = 6$). (b) Time course for TL99 (50 μM) in the absence, (□, $n = 6$) or presence of 0.1 μM nifedipine (■, $n = 6$). Each data point is plotted as mean ± s.e.mean.

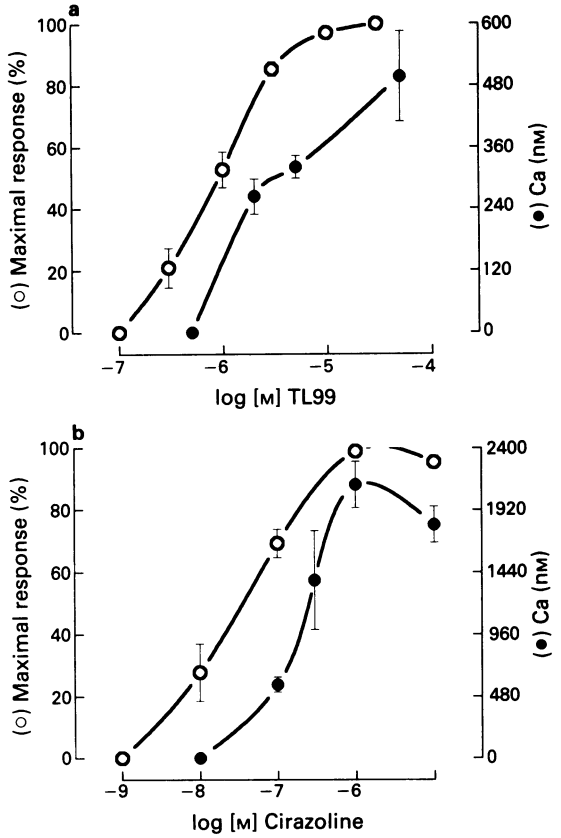

Figure 5 The concentration-dependent changes in intracellular Ca^{2+} stimulated by TL99 and cirazoline in fura-2-loaded freshly dispersed single smooth muscle cells compared with contractile responses to TL99 and cirazoline in artery rings. (a) Concentration-response curves for TL99 in artery ring preparations (○, $n = 4$), and single cells (●, $n = 5-6$). (b) Concentration-response curves for cirazoline in artery ring preparations (○, $n = 4$), and single cells (●, $n = 5-6$). Each data point is plotted as mean ± s.e.mean.

Canada; TL99 [2-(N,N-dimethyl)amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene] from RBI, U.S.A.; yohimbine hydrochloride and phenylephrine hydrochloride from Sigma, U.S.A.; Br-A-23187 (4-bromo A-23187) and fura-2/AM (fura-2-acetoxy methylester) from Molecular Probes Inc., U.S.A. All values are expressed as means ± s.e.mean. Differences between the mean values were determined by Student's t test for unpaired observations, and were regarded as significant when $P < 0.05$.

Results

Studies with single smooth muscle cells

Exposures to cirazoline, phenylephrine and TL99, UK14304 under the conditions described below resulted in >40–50% decreases in length and sometimes blebbing. The average baseline $[Ca^{2+}]_i$ for the smooth muscle cells studied here was 84.5 ± 4.2 nM ($n = 108$). As shown in Figure 1, TL99 (5 μM and 50 μM), in the presence of prazosin (0.05 μM), initiated a slow, dose-dependent increase in $[Ca^{2+}]_i$ and yohimbine (1 μM) inhibited these responses. Perfusion with PSS II alone did not contract the cells and $[Ca^{2+}]_i$ levels remained stable. Figure 2 summarizes the results with cirazoline (0.1 μM and 1 μM), indicating that this selective α_1-adrenoceptor agonist induced a rapid, transient and prazosin-sensitive (0.1 μM) $[Ca^{2+}]_i$ signal.

The time-courses of the response curves for cirazoline and TL99 were different, and the response to TL99, compared with cirazoline, developed more slowly (it took about 20 min to reach maximal response), and resulted in a smaller increase in maximal $[Ca^{2+}]_i$. We also tried different agonists, and found that the time-course response curves induced by phenylephrine (1 μM) and UK14304 (10 μM) were similar to those induced by cirazoline (1 μM) and TL99 (50 μM), respectively (Figure 3). Thus α_1-adrenoceptor agonists produce a fast, comparatively large and transient response, whereas α_2-adrenoceptor agonists induce a slow, sustained and small response. In order to address whether different cellular pathways for Ca^{2+} mobilization were utilized by α_1- and α_2-adrenoceptors, we studied the effects of nifedipine on cirazoline and TL99-induced responses. Figure 4 shows that 0.1 μM nifedipine, almost abolished the increase in intracellular Ca^{2+} induced by 50 μM TL99. In contrast, the same concentration of nifedipine had minimal effects on responses initiated by 1 μM cirazoline.

Studies comparing single cells with ring preparations of rat tail artery

Concentration-response relationships were established for TL99 and cirazoline, and compared to those obtained for contraction of artery rings (organ bath). As shown in Figure 5, TL99 and cirazoline resulted in intracellular Ca^{2+} increases in a concentration-dependent manner. The shapes of concentration-response curves and time-course response curves (data not shown) obtained from freshly dispersed single cells correlated well with those obtained from artery rings.

In single smooth muscle cells, EC_{50} values for cirazoline and TL99 were 1.5×10^{-7} M ($n = 5-6$), and 4.0×10^{-6} M ($n = 5-6$), respectively; in ring preparations, they were $4.3 \pm 1.2 \times 10^{-8}$ M ($n = 4$), and $9.2 \pm 0.9 \times 10^{-7}$ M ($n = 4$), respectively.

Discussion

In the present study, the presence of postjunctional α-adrenoceptors in freshly dispersed single smooth muscle cells from rat rail artery was determined by use of selective α-adrenoceptor agonists and antagonists. Cirazoline, a selective α_1-adrenoceptor agonist (Ruffolo & Waddel, 1982), initiated a $[Ca^{2+}]_i$ signal which was more rapid and transient than that observed for α_2-adrenoceptor agonists. Treatment of the cells with the α_1-adrenoceptor antagonist, prazosin, greatly depressed responses to cirazoline. It can thus be concluded that functional α_1-adrenoceptors are present in freshly dispersed single smooth muscle cells from rat tail artery. Exposure to TL99, an α_2-adrenoceptor agonist (Hicks & Cannon, 1980), resulted in a slowly developing Ca^{2+} signal, that was not affected by the α_1-adrenoceptor antagonist, prazosin (0.05 μM). Treatment of the cells with the α_2-adrenoceptor antagonist, yohimbine greatly inhibited the responses to TL99. The reported K_d and K_i values for prazosin and yohimbine, as determined from radioligand binding studies with crude plasma membrane preparation from the rat tail artery, are 0.17 nM and 1.64 nM, respectively (Cheung & Triggle, 1988), and thus our use of 0.05–0.1 μM prazosin and 1 μM yohimbine should result, respectively, in the inhibition of responses at α_1- and α_2-adrenoceptors.

It is also noted that time-response curves for the fura-2 Ca^{2+} signals following α_1- and α_2-adrenoceptor stimulation were different and that the α_1-mediated Ca^{2+} signal is comparatively fast. Treatment with nifedipine, almost abolished the TL99-induced increase in intracellular Ca^{2+}, but only partially inhibited the cirazoline-induced response. It is clear that activation of α_2-adrenoceptors induces a slow, sustained intracellular Ca^{2+} increase subsequent to the opening of L-type voltage-dependent Ca^{2+} channels, whereas, α_1-adrenoceptors mediate a fast and transient increase in intracellular Ca^{2+}, which is likely to result from the mobilization of intracellular Ca^{2+} and Ca^{2+} influx through receptor-operated channels.

In order to evaluate whether the enzymatic dispersal of the cells results in functional impairment, we compared the concentration and time-course response curves for cirazoline and TL99 obtained from freshly dispersed single cells to those from artery rings. Both the time-course and the concentration-response curves from isolated tissues and single cells were similar. These data suggest that our cell dispersal technique does not result in functional impairment.

These results demonstrate that freshly dispersed vascular smooth muscle cells can be used for the study of signal-transduction pathways coupled to α-adrenoceptor activation. Furthermore, preliminary studies with this technique indicate that, although the activation of either α_1- or α_2-adrenoceptors leads to an increase in $[Ca^{2+}]_i$, the translocation/release processes involved are different.

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