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α-Adrenoceptor stimulated lymphocytes trigger the mechanical response of vas deferens: participation of arachidonic acid metabolites

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1 Normal human lymphocytes (L) (8×10^5 ml^{-1}) incubated with methoxamine (Me) (1×10^{-7} M) (Me-L) triggered the mchanical response of the isolated vas deferens of the rat. L or Me alone did not modify this contractile activity at the concentrations cited above.

2 Me alone (10^{-6} to 10^{-3} M) increased the tension of the vas. In the presence of L (8×10^5 ml^{-1}) the dose-response curve to Me shifted to the left and the efficacy of Me was enhanced.

3 Inhibitors of α_1-adrenoceptors completely blocked the reaction between Me and L while drugs that block α_1 and α_2-adrenoceptors reduced the reaction between Me-L and the vas deferens.

4 Direct contact of Me-L with the assay organ was not necessary. Cell-free supernatants of L exposed to Me (Me-L supernatants) elicited the reaction in the same way as Me-L. This effect required the continuous presence of Me since dialyzed Me-L supernatants were inactive.

5 Inhibitors of lipoxygenase(s) completely blocked the positive inotropic effect of Me-L or of Me-L supernatants. Inhibitors of cyclo-oxygenase potentiated this effect.

6 These results suggest that Me reacts with α_1-adrenoceptors of L. From this reaction, soluble factors are released that potentiate the α-adrenoceptor stimulatory effect of Me on the vas deferens as a consequence of the release of oxidative products of the lipoxygenase/s pathway of arachidonic acid.

Introduction

β-Adrenoceptors have been described in human lymphocytes (Krawietz *et al.*, 1982; Bidart *et al.*, 1983) and the biological effect of binding the specific agonist with the lymphocytes' receptor has been documented in several *in vitro* systems (Chisari & Edgington 1976; Sless & Parrott, 1977).

Human monocytes have been shown to possess α_1-adrenoceptors and their reaction with the corresponding ligand modulates complement component synthesis (Lappin & Whaley, 1982). However, there is little evidence for the presence or function of α-adrenoceptors on lymphoid cells.

The purpose of this work was to investigate whether an α-adrenoceptor agonist could induce some type of activation of the lymphocytes and if α-adrenoceptor-stimulated lymphocytes could in turn react, modifying the physiological responses of other tissues. Vas deferens was selected as an assay organ because it is a smooth muscle particularly rich in α-adrenoceptors.

Compared with the β-adrenoceptor system, the molecular mechanisms involved in α-adrenoceptor responses are not well understood. However, there is much evidence that α-adrenoceptor agonists act through changes in calcium fluxes leading to a rise in cytosolic calcium and stimulate the formation of cyclic GMP (Exton, 1981). Moreover, the hydrolysis of phosphatidylinositol has been postulated as a result of the interaction between the agonists with the adrenoceptors (Michell, 1975; Jones & Michell, 1978; Michell & Kirk, 1981; Farese, 1983). It has also been proposed that metabolites of arachidonic acid could function as intracellular messengers of the α-adrenoceptor system, arachidonic acid being formed by the action of a lipase on diacylglycerol released during phosphatidylinositol breakdown (Exton, 1981).

It appears that arachidonic acid metabolism is intimately related to lymphocyte function. Thus, arachidonic acid (Kelly & Parker, 1979a) or its cyclo-

oxygenase and lipoxygenase products (Kelly *et al.*, 1979b; Parker *et al.*, 1979a; Parker *et al.*, 1979b) were released during the early stages of lymphocyte activation by mitogenic lectins and may control the production and the biological activity of the lymphokine interleukin-2 involved in T cells replication (Henriksen & Frey, 1982).

In this work we demonstrate that normal human lymphocytes can be stimulated by an α-adrenoceptor agonist. Soluble factors released upon exposure of lymphocytes to methoxamine provided a signal to the vas deferens that triggered the generation of lipoxygenase oxidative products from arachidonic acid. These products enhanced the stimulatory reaction between methoxamine and the α-adrenoceptors of the vas deferens.

Methods

Effector cells

Peripheral leukocytes (ML) were obtained by Ficoll Hypaque centrifugation of defibrinated normal human blood (Boyum, 1968). The interphase containing the leukocytes was washed twice with phosphate buffer solution (PBS), once with RPMI 1640 tissue culture medium (Gibco Lab.) buffered with 10 mM HEPES solution (Gibco Lab.) containing gentamycin 50 $\mu g\,ml^{-1}$ (Schering Co Essex Argentina) and 5% heat inactivated foetal calf serum (Gibco Lab.) (RPMI-FCS). The cells were counted in a Neubauer chamber. The leukocytes contained 90–98% mononuclear cells. Leukocytes ($10-20 \times 10^5\,ml^{-1}$) were incubated at 37°C for 18 h in plastic tissue culture bottles (Falcon Plastic) to eliminate adherent cells. The resultant lymphocytes (L) were washed with RPMI-FCS three times and suspended at $8 \times 10^6\,ml^{-1}$ in the same medium. These cells were used in all the experiments as effector cells unless otherwise stated.

When subpopulations of lymphocytes were required, T-lymphocyte-rich and T-lymphocyte-depleted cell fractions were separated by differential centrifugation of E-rosette forming cells (E-RFC) as described previously (Sterin-Borda *et al.*, 1983). E-RFC were mainly T-lymphocytes while interphase lymphocytes (non-ERFC) were composed of non-rosetting T-lymphocytes, B lymphocytes and the contaminant monocytes. L, ERFC and non-ERFC were incubated 16–18 h at 37°C in a 5% CO_2 humidified atmosphere before the reactions. Viability was tested by exclusion of Trypan blue and effector cells containing more than 80% viable cells were used.

Rat isolated vas deferens preparations

Wistar male rats weighing between 200–300 g were killed with a guillotine, their abdomen opened and the vessels and tissues surrounding the vas deferens were carefully dissected. Whole vasa deferentia were gently removed in one piece and placed in Petri dishes containing Krebs-Ringer-bicarbonate solution (KRB) at room temperature, gassed with 5% CO_2 in 95% O_2. Each preparation was cut into three portions: the epididymal, the prostatic and the medial. In the present study we used only the epididymal portion. Before its use the lumen of the vas deferens was flushed several times with KRB solution.

The epididymal portion was suspended in an organ bath containing KRB medium as described by Borda *et al.*, (1981), gassed with a mixture of 95% O_2: 5% CO_2 and maintained at 37°C and pH 7.4. One end of the tissue was anchored to a stationary glass holder and the other to a force transducer (Statham UC-3 Gold Cell). After applying an external tension of 500 mg a 60 min period of equilibration was allowed. No spontaneous activity was detected after equilibrium. Tension (expressed in mg) was recorded with the aid of an ink-writing oscillograph (San Ei - 180).

Concentration-response curves for methoxamine were obtained by the method of Van Rossum (1963).

Supernatants of lymphocytes incubated with methoxamine

Cell-free methoxamine-lymphocyte (Me-L) supernatants were obtained by incubation of 8×10^6 L suspended in 1 ml of RPMI-FCS containing methoxamine 1×10^{-6} M, for 30 min at 37°C; this was followed by centrifugation at 800 *g* for 10 min and the Me-L supernatants were used immediately. Sediment Me-L were washed and resuspened in RPMI-FCS in the absence of Me (washed Me-L). Alternatively, Me-L supernatants were dialyzed for 24 h against PBS to eliminate the adrenoceptor agonist (dialyzed Me-L supernatants).

Drugs

The following were used: methoxamine (Burrough Wellcome CA); phentolamine (Ciba); yohimbine (Sigma Chemical Co); prazosin (Pfizer Lab.); acetylsalicylic acid (ASA) and nordihydroguaiaretic acid (NDGA) (Sigma Chemical Co); indomethacin (Merck, Sharp & Dohme). FPL-55712 (Sodium 7-[3(4-acetyl - 3 - hydroxy - 2 - propylphenoxy) - 2 - hydroxypropoxy]-4 oxo-8-propyl-4H-1-benzopyran-2-carboxylate) was donated by Fisons Ltd.

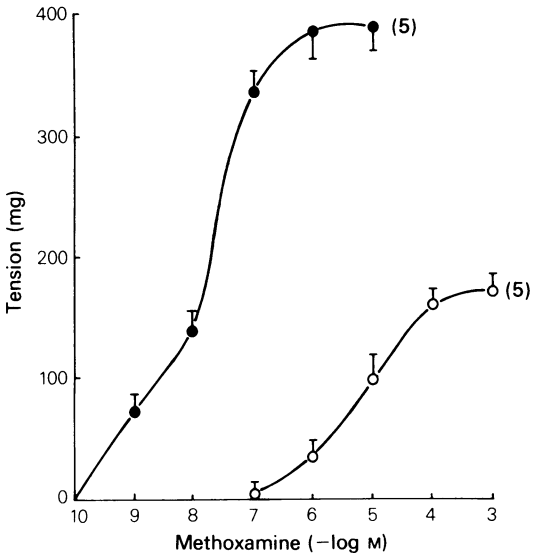

Figure 1 Effect of methoxamine on isolated vas deferens in the presence and in the absence of lymphocytes. Vas deferens were suspended in 10 ml of KRB with 1 ml RPMI containing 8×10^6 lymphocytes (L) ml^{-1} (●) or 1 ml RPMI (○); increasing amounts of methoxamine (Me) were added. S.e.mean shown by vertical lines. The number of experiments is indicated in parentheses.

Statistical analysis

All results are expressed as means ± s.e.mean. The differences between means were calculated using Student's *t* test and were considered significant if *P* was equal to 0.05 or less.

Results

Dose-response curves to methoxamine in the presence or absence of lymphocytes

Figure 1 shows dose-response curves to methoxamine (Me) on the tension of vas deferens in the presence and in the absence of lymphocytes (L). It can be seen that in the range of 10^{-6} to 10^{-3} M, Me increased the tension. In the presence of 8×10^6 L ml^{-1}, the dose-response curve shifted to the left. Preincubation of vas deferens with phentolamine (10^{-7} M) and prazosin (10^{-7} M) abolished, but yohimbine (10^{-7} M) partially inhibited the stimulatory action of Me in the presence of L (Table 1).

Table 1 Influence of α-adrenoceptor antagonists on the inotropic positive effect of methoxamine plus lymphocytes (Me-L)

Incubation (30 min) *of vas deferens with*	*Reagents*	*Tension** (mg)	n
KRB alone	Me-L	325 ± 11	6
KRB + phentolamine (10^{-7} M)	Me-L	0	4
KRB + prazosin (10^{-7} M)	Me-L	25 ± 4	5
KRB ± yohimbine (10^{-7} M)	Me-L	168 ± 8	6

* Mean ± s.e.mean. Methoxamine-treated lymphocytes (Me-L) were added to the organ bath at a final concentration of 8×10^5 L ml^{-1} and 10^{-7} M Me.
n = number of preparations.

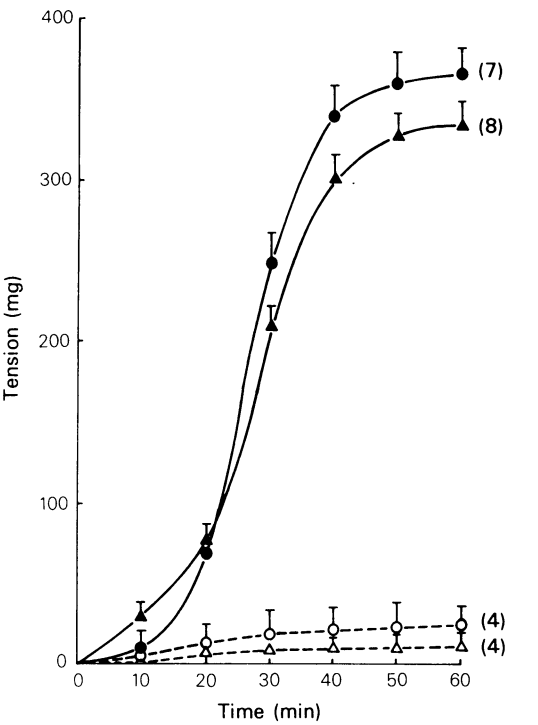

Figure 2 Time course of the reaction of methoxamine-treated lymphocytes and the corresponding supernatants with the vas deferens. Vasa deferentia were suspended in 10 ml of KRB. The organ bath was supplied with 1 ml of methoxamine (10^{-6} M) in RPMI (○); 1 ml of 8×10^6 lymphocytes (L) ml^{-1} suspension (△); 1 ml of 8×10^6 L ml^{-1} that had been preincubated for 30 min at 37°C with RPMI containing 10^{-6} M methoxamine (▲) or 1 ml of supernatants from the reaction of 8×10^6 L ml^{-1} and 10^{-6} M methoxamine (●). Details and conditions as in Figure 1.

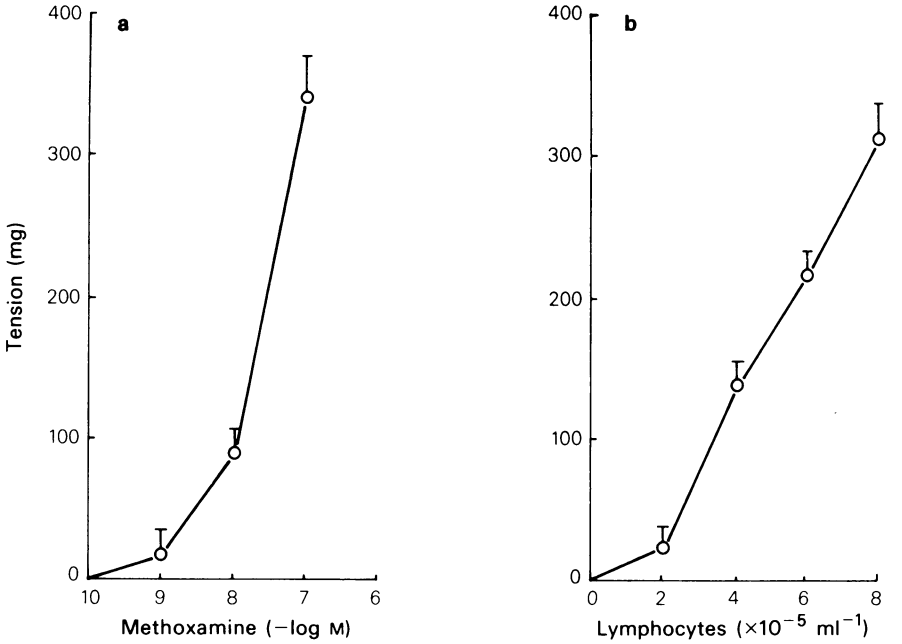

Figure 3 Effect of various methoxamine concentrations in the presence of a fixed amount of lymphocytes and of changing the lymphocyte concentration at a subthreshold dose of methoxamine. (a) Dose-response curve to methoxamine (Me) in the presence of 8×10^5 L ml^{-1}, (b) dose-response curve to L in the presence of 10^{-7} M Me. The changes in tension were calculated after the addition of L that had been preincubated with the corresponding doses of Me for 30 min at 37°C in the organ bath. Other details as in Figure 1. Values of 4 experiments in each group.

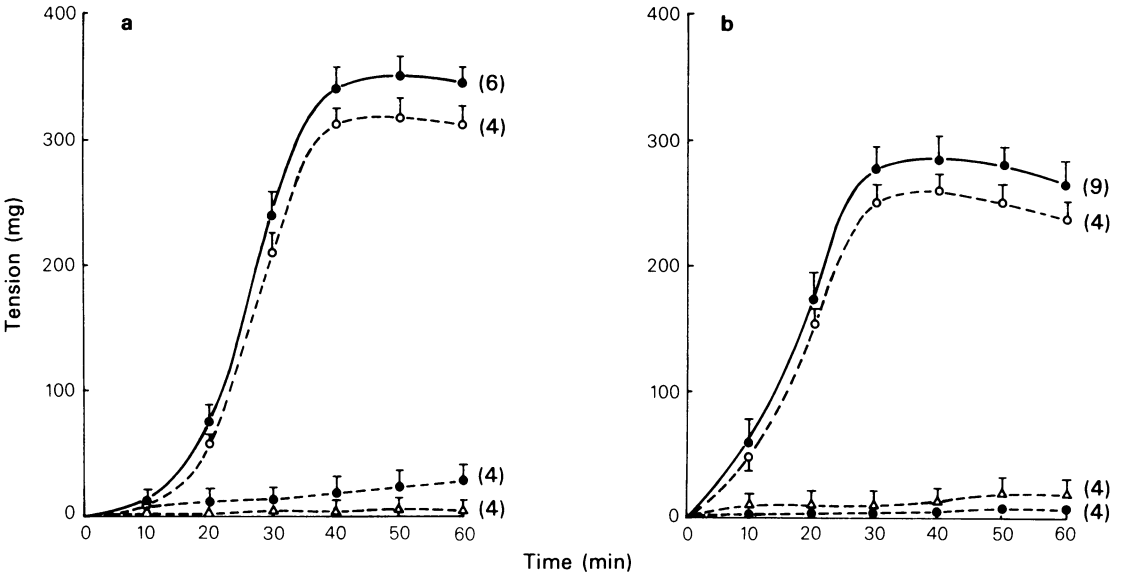

Figure 4 Influence of α-adrenoceptor antagonists on the reaction of normal human lymphocytes (L) with methoxamine (Me). (a) Reaction of Me-L or of α-adrenoceptor antagonist-treated Me-L and (b) the reaction of the corresponding supernatants on vas deferens. α-Adrenoceptor antagonists: 10^{-7} M phentolamine (●---●); 10^{-7} M prazosin (△--△) and 10^{-7} M yohimbine (○---○) or KRB (●—●) were incubated with 8×10^6 L ml^{-1} for 20 min at 37°C before addition of 10^{-6} M Me. Final concentration of Me in the organ bath was 10^{-7} M. Other details as in Figure 1.

Effect of lympocytes treated with methoxamine on the tension of vas deferens

Figure 2 shows that either 8×10^5 L ml^{-1} or 1×10^{-7} M Me added alone to vasa deferentia did not trigger mechanical activity. In contrast, when L were preincubated at 37°C for 30 min with Me (Me-L) they triggered the mechanical activity of the vas deferens. The supernatants of the reaction between Me and L were collected and added to the organ bath. The results shown in Figure 2 demonstrate that soluble factors derived from the interaction of Me with L, could replace the intact L. The effects of Me-L or the Me-L supernatants developed with time and were maximal after 50 min of the reaction with the organ.

The magnitude of stimulation was directly proportional to the concentration of Me or effector cells in the medium (Figure 3).

To determine whether α-adrenoceptors were involved in the reaction between Me and L, the effector cells were incubated for 20 min with α-adrenoceptor blocking agents before exposure to Me. The results shown in Figure 4 indicate that the inhibition of L α_1-adrenoceptors with phentolamine or prazosin efficiently blocked the positive inotropic effect of both Me-L and Me-L supernatants on vasa deferentia. In contrast, inhibition of L α_2-adrenoceptors with yohimbine did not block these stimulatory effects.

The continuous activation of α-adrenoceptors of the vas deferens by Me appears to be a requisite to trigger the reaction. Table 2 supports this statement,

Table 2 Effects of washed methoxamine-stimulated lymphocytes (Me-L) and dialyzed Me-L supernatants on the tension of the vas deferens

	Reagents	*Tension* (mg)	n
	Mel-L*	351 ± 14	6
A	Washed Me-L**	0	6
	Washed Me-L plus Me*	345 ± 16	6
	Me-L supernatants*	359 ± 10	7
B	Me-L dialyzed supernatants**	0	7
	Me-L dialyzed supernatants plus Me*	326 ± 13	7

The organ bath was supplied with: (A) 1 ml of RPMI-FCS containing 8×10^6 L that had been incubated for 30 min at 37°C with 10^{-6} M Me (Me-L); 1 ml Me-L washed once with 5 ml RPMI-FCS and resuspended in 1 ml Me free RPMI-FCS (washed Me-L); 1 ml washed Me-L plus 10^{-6} M Me (washed Me-L plus Me). (B) 1 ml supernatants from Me-L (Me-L supernatants); 1 ml Me-L supernatant dialyzed against PBS for 24 h (dialyzed Me-L supernatants); 1 ml dialyzed Me-L supernatants plus 10^{-6} M Me.

**The final concentration of Me in the organ bath was nil or * 10^{-7} M.

n = number of preparations tested.

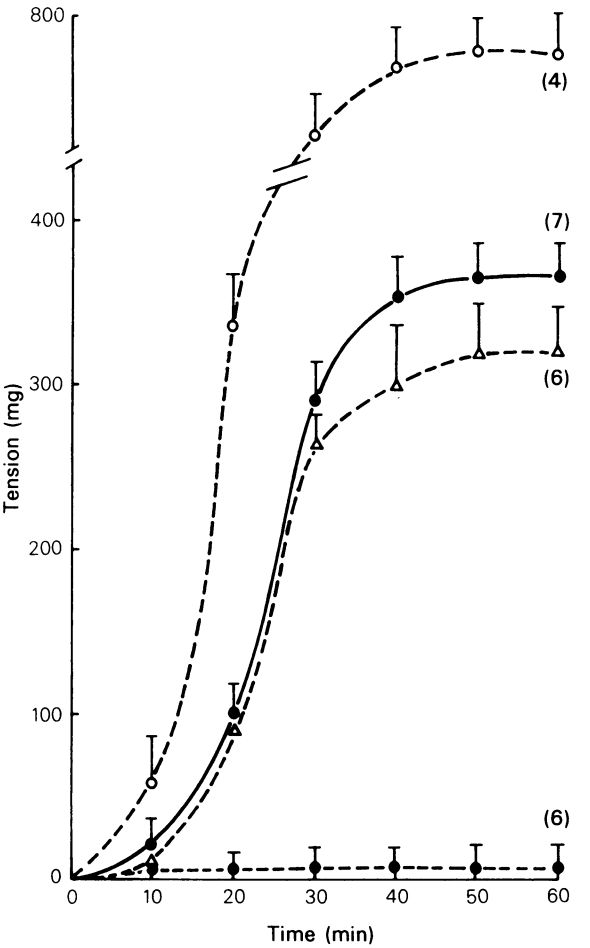

Figure 5 Reaction of methoxamine-treated normal human lymphocytes in the presence of inhibitors of arachidonic acid metabolism. The inhibitors: indomethacin (10^{-6} M) (○---○); nordihydroguaiaretic acid (NDGA) (10^{-5} M) (●---●) or FPL-55712 (10^{-7} M) (△---△), were added to the organ bath 20 min before addition of Me-L (●—●). Other details as in Figure 1.

because washed Me-stimulated L (washed Me-L) or dialyzed supernatants from Me-L (dialyzed Me-L supernatants) were unable to induce any activity, but this was restored by the presence of 10^{-7} M of Me.

Mechanisms of the reaction of methoxamine plus lymphocytes (Me-L) with vas deferens

Several inhibitors acting on different pathways that could result in stimulation of tension of the vas deferens were used, in order to determine the nature of the mechanisms triggered by Me-L.

Table 3 Effect of supernatants from the reaction of lymphocytes (L) and methoxamine (Me) on the tension of the vas deferens in the presence of inhibitors of arachidonic acid oxidative metabolism

Supernatants	*Vas deferens*	*Tension* (mg)	n
L	KRB	0	5
Me-L	KRB	325 ± 15	6
Me-L	KRB + NDGA	20 ± 5	5
Me-L	KRB ± dithizone	0	5
Me-L	KRB + FPL-55712	312 ± 10	4
Me-L	KRB + Indo	710 ± 32	5
Me-L	KRB ± ASA	698 ± 28	4

Supernatants of 8×10^6 L incubated with 1×10^{-6} M methoxamine (Me) (Me-L) for 30 min at 37°C were added to the vas deferens suspended in KRB or that had been previously incubated with 10^{-5} M nordihidroguaiaretic acid (NDGA); 10^{-5} M dithizone; 10^{-7} M FPL-55712; 10^{-6} M indomethacin (Indo) or 1.8×10^{-4} M acetysalicylic acid (ASA) for 30 min. Mean ± s.e.mean.
n = number of preparations.

As shown in Figure 5, the inhibition of the cyclooxygenase activity of the vas deferens by indomethacin (Indo) or acetylsalicylic acid (ASA) (data not shown) did not block but actually stimulated the effect of Me-L. On the other hand, nordihydroguaiaretic acid (NDGA), inhibitor of the lipoxygenase/s pathway of arachidonic acid metabolism, interfered with the reaction of Me-L on the vas deferens. In contrast, FPL-55712, an inhibitor of slow reacting substance (SRS-A), did not modify this effect.

Me-L supernatants were assayed on vasa deferentia that had been incubated with lipoxygenase inhibitor and they behaved in a similar way to intact L (Table 3). Thus, the effect of Me-L or of the supernatants required active lipoxygenase/s enzymes from the vas deferens, since it did not occur when the organ had been previously treated with NDGA. Cyclooxygenase products were not the mediators of the inotropic effect of Me-L.

Table 4 Characterization of the effector cells in the reaction between lymphocytes plus methoxamine and vas deferens

Effector cells plus methoxamine	*Tension** (mg)	n
T-lymphocyte rich	342 ± 18	4
T-lymphocyte depleted	385 ± 20	4

The T-lymphocyte rich (ERFC) and T-lymphocyte depleted (non-ERFC) populations were prepared as described in methods. *Mean ± s.e.mean values of tension (mg) developed after 50 min of incubation of vas deferens with 8×10^5 ml^{-1} effector cells plus methoxamine 10^{-7} M. n = number of preparations.

Characterization of the effector cells

To determine if lymphocytes of different lineage could be involved in the reaction with isolated vas deferens, different L populations were used.

The results shown in Table 4 indicate that both ERFC and non-ERFC were equally reactive with the vas deferens in the presence of Me.

Discussion

The results of this work demonstrate that methoxamine (Me) reacts *in vitro* with normal human lymphocytes (L) and that the products of the α-activated lymphocytes potentiate the α-adrenoceptor-mediated contractile response of the vas deferens. Moreover, the positive inotropic effect of Me itself was potentiated by the presence of L in the incubation media.

The reaction between Me, L and the vas deferens involved many steps: (1) the action of Me on the L; (2) the production of soluble factors from L and (3) the mechanical response of the vas deferens.

The first step, that is concerned with the action of Me on L, appears to involve specific α_1-adrenoceptors, because phentolamine and prazosin, efficiently blocked the reaction and yohimbine had no effect (Langar, 1981).

Results with cell-free supernatants of Me plus L (Me-L) indicate that upon exposure of L to Me, soluble factors were released from L and that these factors could enhance the effect of Me on the vas deferens. Hence, close contact between L and the vas deferens was not necessary.

Contraction of the vas deferens resulted from its reaction with Me-L or Me-L supernatants and increased tension developed soon after exposure to Me-L.

The fact that, washed lymphocytes or dialyzed supernatants were unable to induce any activity, while this was restored by the addition of Me, indicates that the continuous presence of Me on the surface of the vas deferens is a requisite for stimulation. The effect of L or their soluble factors would be to enhance the reaction between Me and the α-adrenoceptors on the vas deferens. In this way Me could react on α-adrenoceptors of lymphocytes and on the α-adrenoceptors of vasa deferentia, but on L, it would recognize only the α_1-subtypes while on the vas deferens it would react both with α_1 and α_2-adrenoceptor subtypes.

The fact that inhibitors of lipoxygenase/s such as NDGA and dithizone (Pistorious & Axelrod, 1974, Hamberg & Samuelsson, 1974; Hamberg, 1976) prevented the reaction, indicates that metabolites of arachidonic acid via this catalyzed pathway are also involved. However, the fact that inhibition of the vas deferens' lipoxygenase/s pathway prevents the stimulatory effect of both Me-L and Me-L supernatants stresses the role of the enzymatic system from the assay organ in the production of the active arachidonic acid metabolites.

On the other hand, inhibitors of cyclo-oxygenase activity such as Indo or ASA (Vane, 1971) lead to an enhancement of tension; this may reflect reduction of the generation of some prostanoids that inhibit contractility or a diversion of arachidonic acid into the lipoxygenase pathway with increased production of positive inotropic substances.

Recently we have demonstrated that normal human lymphocytes activated by a mitogenic lectin or by a calcium ionophore can react *in vitro* with rat isolated atria increasing both the tension and the frequency of contractions by mechanisms involving the release of an active substance derived from arachidonic acid via the lipoxygenase catalyzed pathway (Sterin-Borda *et al.*, 1983). It was later demonstrated that sodium arachidonate could trigger the reaction between normal lymphocytes and rat isolated atria, replacing the initial steps provided by lectin stimulation of the lymphocytes membrane. In this reaction soluble factors could trigger the positive inotropic and chronotropic responses (Borda *et al.*, 1983).

We explored the action of Me plus L on rat isolated atria and positive and chronotropic effects were also observed (Sterin-Borda, unpublished observation). But in contrast to the above mentioned results the reaction between Me, L and the assay organ did not involve SRS-A, since FPL-55712 (Burka & Paterson, 1981) was ineffective.

To obtain information about the subpopulations of lymphocytes that were able to react with Me, we separated fractions containing predominantly T lymphocytes (ERFC) from those rich in non-T cells (non-ERFC) (Sterin-Borda *et al.*, 1983). the stimulatory effect observed with Me was recovered with both subsets of lymphocytes, indicating that there was no subtype specificity in the reaction. These results contrast with those described for human normal lymphocytes activated with mitogenic lectins, in which cells of the T lineage were responsible for the positive inotropic and chronotropic effects (Sterin-Borda *et al.*, 1983).

We propose that upon exposure of lymphocytes to Me, the α-adrenoceptor agonist 'recognized' α_1-adrenoceptors on the lymphocyte membrane. Soluble factors were released by the reaction and they in turn influenced the effect of Me on the vas deferens, triggering the lipoxygenase/s oxidative pathway.

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