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RYANODINE AS INHIBITOR OF CHEMOTACTIC PEPTIDE-INDUCED CHEMOTAXIS IN HUMAN NEUTROPHILS

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Abstract—Ryanodine gave a moderate inhibition of chemotactic peptide-activated chemotaxis by intact human neutrophils. Chemotaxis by electroporated neutrophils was strongly inhibited in the nanomolar concentration range. Inhibition of chemotaxis by electroporated neutrophils occurs at concentrations known to open calcium channels in ryanodine-sensitive Ca^{2+} stores. Whereas migration by formyl-methionyl-leucyl-phenylalanine (fMLP)- or interleukin-8-activated electroporated neutrophils was strongly inhibited by ryanodine, chemotaxis induced by protein kinase C activators was not affected. This suggests that the importance of ryanodine-sensitive Ca^{2+} stores for migration depends on the type of activator used. Ryanodine gave an increase of cytoplasmic free calcium due to the liberation of calcium from internal stores and to the influx of extracellular calcium. The results show that the neutrophil contains ryanodine-sensitive calcium stores that might be involved in receptor-mediated chemotaxis.

Key words: ryanodine; neutrophil; chemotaxis; calcium; electroporated neutrophils

The plant alkaloid ryanodine is a specific probe for a certain type of calcium channel in the sarcoplasmic reticulum of skeletal and cardiac muscle cells [1]. It has two distinct effects: at low (nanomolar range) concentrations ryanodine opens the channel, whereas at higher (micromolar) concentrations the calcium channel is blocked [2, 3]. Recently it has become evident that ryanodine receptors are also present in cells having less well-defined calcium stores. Ryanodine inhibited the caffeine-induced increase of cytoplasmic Ca^{2+} concentration in adrenal chromaffin cells [4]. Catecholamine secretion from chromaffin cells was inhibited by ryanodine via an inhibition of Ca^{2+} mobilization [5]. Vasopressin-induced Ca^{2+} spikes in hepatocytes were inhibited by ryanodine, though the drug had no effect on phenylephrine-induced Ca^{2+} oscillations [6]. The binding of ryanodine to liver microsomal fractions differed from that to sarcoplasmic reticulum with regard to Ca^{2+} dependence and caffeine sensitivity [7].

It is thought that neutrophils possess intracellular Ca^{2+} stores that resemble the sarcoplasmic reticulum of muscle cells [8, 9]. The properties and importance of these calciosomes in neutrophil functions are largely unknown. There are some indications that Ca^{2+} from these stores plays a role in neutrophil migration. While migration of neutrophils proceeds in the absence of extracellular calcium, and no changes in cytoplasmic calcium are required for fMLP†-activated chemotaxis, migration is strongly inhibited when the cells are depleted of all cellular calcium [10]. Furthermore, several calcium channel blockers completely inhibit migration of electroporated neutrophils in the nanomolar concentration range [11]. The latter observation supported the view that calcium derived from calcium-storing organelles played a role in neutrophil migration.

Migration of neutrophils represents an important property of these cells, and is of relevance both for the defense mechanism of the body against foreign invaders and for the pathogenesis of inflammatory conditions. The present study was undertaken to determine whether ryanodine had an effect on chemotaxis and cytosolic calcium of human neutrophils. Both intact and electroporated neutrophils were used, because in a previous study [11] we observed that direct application of certain agents to the cytoplasm resulted in an effect at concentrations several orders of magnitude lower than those required for the same effect in intact cells.

MATERIALS AND METHODS

Isolation of neutrophils

Human neutrophils were isolated from the venous blood of healthy volunteers by dextran sedimentation followed by centrifugation over Ficoll-Isopaque, and hypotonic hemolysis of contaminating erythrocytes. Isolated neutrophils were resuspended in a medium containing 140 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes (pH = 7.3), and 0.5% bovine serum albumin. During the experiments 1 mM Ca^{2+} and 1 mM Mg^{2+} were included in the medium, unless otherwise indicated. The final cell concentration during the experiments was 3×10^6 neutrophils per mL.

Migration measurements

Cell migration was measured with the Boyden chamber technique, as described by Boyden [12], and modified by Zigmond and Hirsch [13]. The two compartments of the chamber were separated by a cellulose acetate Millipore filter with a pore size of 3 μm. Chemotaxis was activated by the chemotactic peptide fMLP, which was placed in the lower compartment at a concentration of 10^{-9} M. Neutrophils were placed in the upper compartment of the chamber, followed by incu-

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† Abbreviations: fMLP, formylmethionyl-leucyl-phenylalanine; PMA, phorbol myristate acetate; IL8, interleukin-8; diC8, dioctanoyl glycerol; TNF, tumor necrosis factor; Ca_i, concentration of cytoplasmic free calcium.

bation for 35 min at 37°C. After migration, the filters were fixed and stained, and the distance travelled in micrometers into the filter was determined according to the leading front technique [13]. The assays were carried out in triplicate, and the migration distance of the neutrophils determined at five different filter sites.

Determination of intracellular calcium

Neutrophils (1×10^7 cells per mL) were incubated with 1 μM Fura-2/AM for 30 min at 37°C in the presence of 1 mM Ca^{2+}. After washing, the cells were resuspended in medium and used at a concentration of 3×10^6 cells per mL. Fura-2 fluorescence was measured in a Perkin Elmer LS50B fluorescence spectrophotometer equipped with a temperature-controlled cuvette compartment and a mixing device. Fluorescence (emission wavelength 510 nm) was recorded at two excitation wavelengths (340 nm and 390 nm), and the data used to calculate the concentrations of cytoplasmic free calcium. Traces given are representative for three different experiments.

Electroporation of neutrophils

Neutrophils were electroporated according to the method of Grinstein [14], with minor modifications [15]. The electro-permeabilization procedure was carried out at room temperature. When permeabilization was carried out at 0°C the cells were not able to migrate. Neutrophils (3×10^6 per mL) in permeabilization medium (135 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes pH 7.0, 10 mM glucose and 0.5% BSA) and reagents as indicated were placed in the cuvette of a BioRad Gene Pulser. The cells were exposed to two discharges of 4.75 kV/cm from a 25 μF capacitor. Between the two discharges, the cell suspension was stirred with a plastic pipette. After permeabilization and mixing, 0.2 mL of the suspension was placed in the upper compartment of the Boyden chamber. Because the cells start to close after a few minutes at room temperature, ryanodine was included in the medium during electroporation. Unless otherwise indicated, 1 mM Ca^{2+} was included in the medium before electroporation, because this concentration of Ca^{2+} did not require the use of calcium buffers, and migration is optimal at this concentration.

Statistical analysis

Three or four separate experiments were performed with cells of different donors. Chemotactic assays were carried out in triplicate, and the migration distance of the neutrophils was determined at five different filter sites. Values given are arithmetical means ± standard error of the mean of the separate experiments. Significances were calculated with Student's *t*-test; a value of $P < 0.01$ was considered as statistically significant.

RESULTS

Chemotaxis activated by fMLP in intact cells was moderately inhibited by ryanodine. Inhibition increased up to a concentration of 4 μM ryanodine where inhibition was 20%; higher concentrations gave no additional inhibition (Fig. 1). Electroporated cells were able to migrate if permeabilization was carried out at room temperature; both random migration and fMLP-activated migration were, however, somewhat reduced. Migration of electroporated neutrophils activated by fMLP was strongly inhibited by ryanodine, inhibition being complete (nearly to the level of random migration) at a concentration of 1 μM ryanodine (Fig. 1). Inhibition of migration by electroporated neutrophils by ryanodine was slightly higher in the absence of calcium than with calcium present, but the difference was small (26% inhibition in the presence of EDTA and 22% inhibition in the presence of 1 mM Ca^{2+}) (Table 1). Though it could be that added Ca^{2+} antagonized the action of ryanodine, the effect is too small to permit firm conclusions to be drawn.

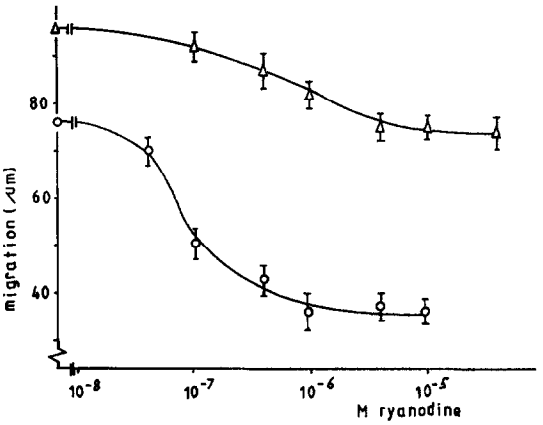

Fig. 1. The effect of increasing concentrations of ryanodine on fMLP-activated migration of intact neutrophils (——△——), or electroporated neutrophils (——○——). Random migration (in the absence of fMLP) was 47.1 ± 2.1 μm for intact cells, and 33.6 ± 2.0 μm for electroporated cells.

Chemotaxis may be induced by receptor-mediated processes such as fMLP- or IL8-induced chemotaxis. Apart from receptor-mediated activation of neutrophil migration, it is possible to induce chemotaxis in neutrophils with agents that are activators of protein kinase C, such as dioctanoyl glycerol [16], PMA [17, 18], and R59022 [17]. The latter substance is an inhibitor of diacylglycerol kinase, and thus induces accumulation of diacylglycerol, a natural activator of protein kinase C. Inhibition of chemotaxis did not occur with all types of chemoattractants. Only fMLP-activated chemotaxis and—to a somewhat lesser degree—IL-8 activated chemotaxis were inhibited by ryanodine. Activation of electroporated neutrophils by agents that act via the protein

Table 1. The effect of calcium on ryanodine-induced inhibition of fMLP-activated migration by electroporated neutrophils

	Migration (μm)	
	–	+0.4 μM ryanodine
EDTA	63.1 ± 2.4	45.9 ± 1.9
EGTA	66.3 ± 2.7	48.9 ± 2.1
Ca^{2+}, 1 μM	66.8 ± 2.2	50.9 ± 1.9
Ca^{2+}, 1 mM	70.1 ± 2.1	54.8 ± 1.7

During incubation with EDTA, no Mg^{2+} was present, whereas in all other cases 1 mM Mg^{2+} was present in the medium. In the lower compartment of the Boyden chamber, 10^{-9} M fMLP was present. Random migration in the presence of 1 mM Ca^{2+} was 31.9 ± 2.0 μm and, in the presence of 1 mM EDTA, 28.0 ± 2.1 μm. Inhibition by ryanodine is significant in all cases ($P < 0.001$).

kinase C pathway—phorbol myristate acetate, dioctanoyl glycerol, and R59022—was not inhibited by ryanodine (Table 2).

Preincubation of neutrophils with ryanodine caused a time-dependent loss of inhibitory potency, both in intact and electroporated cells. Loss of potency occurred after 10–20 min contact with ryanodine (Fig. 2). Addition of ryanodine after preincubation for 20 min with ryanodine did not result in a longer inhibition. The loss of inhibitory potency was not dependent on the presence or absence of Ca^{2+} during preincubation (Table 3).

Caffeine inhibited fMLP-activated chemotaxis, in line with previous findings by other investigators [19]. Inhibition by ryanodine and caffeine was about additive when both agents were present simultaneously (Table 4). Ryanodine gave some additional inhibition when cells were preincubated with caffeine for 20 min (Table 4).

Ryanodine caused an increase in cytoplasmic free calcium. In the absence of extracellular Ca^{2+}, ryanodine also caused an increase in Ca^{2+}_i, but the increase was smaller than with extracellular calcium present (Fig. 3). Caffeine had little effect on the level of Ca^{2+}_i. Ryanodine gave no increase in Ca^{2+}_i when the cells were preincubated with caffeine. Addition of fMLP to neutrophils after preincubation with caffeine gave the same increase of Ca^{2+}_i as in the absence of pretreatment (Fig. 3). When ryanodine was added to cells preincubated with ryanodine for 20 min, the response was less than with no pretreatment (Fig. 3).

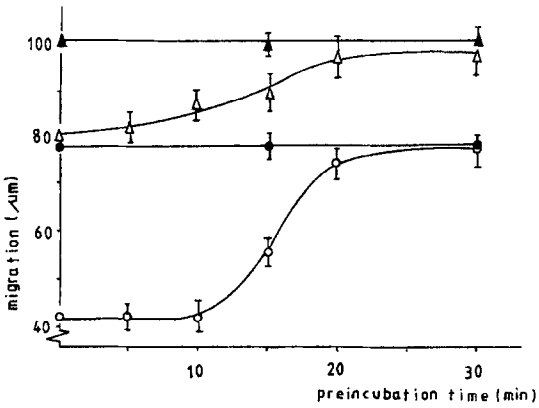

Fig. 2. The effect of variable preincubation time with ryanodine on migration of fMLP-activated neutrophils. Intact cells were preincubated with 4 µM ryanodine (——△——) or without ryanodine (——▲——), and electroporated cells were preincubated with 0.4 µM ryanodine (——○——) or without ryanodine (——●——), for the time indicated, at 37°C. Subsequently, the cells were placed in the upper compartment of the Boyden chamber, and incubated as described in Methods.

DISCUSSION

The results show that the neutrophil contains ryanodine-sensitive calcium stores, and that association of ryanodine with the neutrophil results in inhibition of chemotactic migration. The effect is most pronounced with electroporated cells, indicating that in analogy with the calcium channel blockers verapamil and nifedipine, ryanodine acts on the cell interior rather than the plasma membrane. The ryanodine-induced release of calcium from ryanodine-sensitive stores coincides with inhibition of fMLP- or IL-8-activated chemotaxis. Though a comparison between these processes is hampered by the fact that migration occurs in a three-dimensional system with adherent cells, and changes in cytoplasmic free calcium were measured in suspension, it seems possible that there may be a relationship between these processes. In that event the calcium stores seem to have a differential importance for migration, one dependent on the type of activator. Ryanodine-sensitive calcium stores have no role in chemotaxis induced by activators of protein kinase C, for ryanodine has no effect on migration induced by these activators.

At present, two opposing effects of ryanodine are known: Low ryanodine concentrations open the calcium channel of the stores of the sarcoplasmic reticulum, and high concentrations close the channel [2, 3]. The closing of verapamil-sensitive calcium channels in the membrane of calcium stores of neutrophils has been shown to inhibit migration [11]. However, the inhibitory effect of ryanodine on migration occurs at concentrations that correspond with the activating concentrations in the sarcoplasmic reticulum. The observation that ryanodine causes a release of calcium from intracellular stores at concentrations that inhibit migration suggests that inhi-

Table 2. Inhibition of migration by electroporated neutrophils, activated by several types of activators, by ryanodine

Activator	Migration (µm)	
	–	+ 0.4 µM ryanodine
fMLP	74.8 ± 2.1	48.3 ± 2.1
IL8	68.0 ± 1.9	53.1 ± 1.9
PMA	68.5 ± 1.9	68.8 ± 2.3
R59022	85.1 ± 2.3	85.0 ± 2.1
diC8	85.2 ± 1.9	84.9 ± 1.9

Neutrophils were electroporated and placed with or without 0.4 µM ryanodine in the upper compartment of the Boyden chamber. In the lower compartment, 10^{-9} M fMLP, 5×10^{-9} M interleukin 8 (IL8), 100 pM phorbol myristate acetate (PMA), 50 µM R59022, or 50 µM dioctanoyl glycerol (diC8) was present as an activating agent. Under these conditions, random migration was 34.8 ± 2.3 µm. Inhibition by ryanodine was significant with fMLP or IL-8 as chemotactic agents ($P < 0.001$), but was not statistically significant for PMA, R59022, and diC8.

Table 3. Effect of preincubation with ryanodine on inhibition of fMLP-activated chemotaxis

Present during preincubation	Added after preincubation	Migration (µm)
–	–	71.8 ± 2.3
–	Ryanodine, Ca^{2+}	44.6 ± 1.8*
Ryanodine, Ca^{2+}	–	71.4 ± 1.9
Ryanodine, Ca^{2+}	Ryanodine, Ca^{2+}	70.9 ± 2.2
EGTA	Ca^{2+}	71.5 ± 2.3
Ryanodine, EGTA	Ca^{2+}	70.5 ± 2.1

Neutrophils were preincubated with or without 0.4 µM ryanodine, electroporated, and placed with or without ryanodine in the upper compartment of the Boyden chamber. In the lower compartment, 10^{-9} M fMLP was present. Under these conditions, random migration was 33.4 ± 2.1 µm. Ca^{2+}: 1 mM; EGTA: 50 µM. *$P < 0.001$.

Table 4. The effect of caffeine and ryanodine on migration by fMLP-activated neutrophils

	Migration (μm)			
	Intact		Electroporated cells	
	–	+	–	+ ryanodine
No preincubation				
–	91.6	76.9	66.2	31.9
25 mM Caffeine	50.9	35.9	32.5	29.3
Preincubation with caffeine for 20 min				
–	93.2	78.0	68.1	33.2
25 mM Caffeine	33.8	29.7	25.0	21.3

In the lower compartment of the Boyden chamber, 10^{-9} M fMLP was present. SEM varied from 1.4 to 2.5 μm. Random migration was 46.3 ± 1.9 μm for intact cells, and 32.7 ± 2.1 μm for electroporated cells.

bition of migration corresponds with an activating effect of ryanodine on calcium stores. It thus seems unlikely that ryanodine inhibits migration by closing calcium channels, the way in which verapamil, nifedipine, and nitrendipine inhibit migration.

The possibility that ryanodine inhibits migration by opening the calcium channel and that, consequently, the enhancement of Ca_i is a cause of inhibition seems contradictory. We have shown that migration of electroporated cells occurs in the presence of (high) concentrations of calcium [11]. Furthermore, fMLP and other chemotactic agents cause an increase in Ca_i. However, there are a few observations that suggest that a (local) enhancement of calcium might somehow be detrimental to chemotaxis, especially receptor-mediated rather than protein kinase C-mediated chemotaxis. The migration of electroporated cells is less than that of intact cells, but there exists a difference between receptor-mediated and protein kinase C-mediated chemotaxis. In a representative experiment, fMLP-activated chemotaxis was 101.0 ± 2.2 μm for intact cells and 73.8 ± 1.7 μm for electroporated cells. The decrease was much less when R59022 was used as a chemotactic agent: Here, intact cells migrated 87.5 ± 1.5 μm, and electroporated cells 83.3 ± 2.2 μm. Apparently, there is a factor—possibly calcium influx—that is more injurious for fMLP-induced chemotaxis than R59022-induced chemotaxis. The fMLP concentration used to enhance Ca_i is mostly 1 μM, and this concentration gives no stimulation of migration [21]. At a concentration of 10^{-9} M, fMLP gives only a slight increase in Ca_i. A number of agents give an increase in Ca_i, stimulate the respiratory burst (mostly via priming), and inhibit migration. Among these are hexachlorocyclohexane [22], ATP [20, 23], cytochalasin B [24, 25], ionophore A23187 [11], and TNF [26], all at specific concentrations. Apart from cytochalasin B, which interferes with actin polymerization, the mechanism of inhibition by these substances is obscure. It seems possible that under certain circumstances, an enhancement of Ca_i, though favorable for the respiratory burst, is detrimental to receptor-mediated chemotaxis. The arguments presented provide circumstantial rather than conclusive evidence that the first step in ryanodine-induced inhibition of migration is the result of an unregulated increase in cytoplasmic free calcium. There remain, however, a number of uncertainties. Notably, the change of calcium in electroporated cells remains obscure, because during stimulation of calcium release the cells are open. It is possible that the inhibition is initiated by a local change of calcium rather than a general enhancement of cytosolic free calcium. Furthermore, it cannot be excluded that ryanodine inhibits migration via a mechanism not related to its effect on calcium stores.

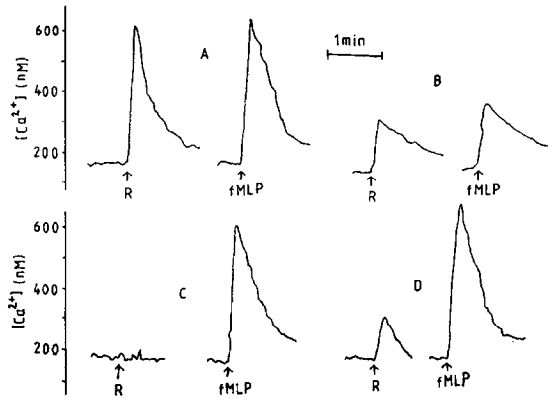

Fig. 3. The effect of ryanodine (4 μM) on cytoplasmic free calcium. The experiments were performed with intact neutrophils. As a comparison, the effect of fMLP (10^{-6}M) is included: A: control (1 mM Ca^{2+} present); B: 1 mM EGTA (no extracellular Ca^{2+}); C: pretreatment of cells with 25 mM caffeine for 20 min; D: pretreatment of cells with ryanodine (4 μM) for 20 min (C and D: 1 mM Ca^{2+} present). At the time indicated with an arrow, ryanodine (R) or fMLP was added.

In several cell types (smooth muscle cells, adrenal chromaffin cells, neuronally-derived pheochromocytoma cells), ryanodine is thought to interfere with calcium release from caffeine-sensitive stores. In these cells ryanodine counteracts the effect of caffeine. Though the complete inhibition of the ryanodine response on intracellular calcium by caffeine suggests that caffeine acts on ryanodine-sensitive Ca^{2+} stores, the use of caffeine as an antipode of ryanodine in neutrophils is complicated by the fact that caffeine itself inhibits neutrophil locomotion. This effect has been observed previously, and it was suggested that the inhibition was due to a caffeine-induced accumulation of cAMP [19]. In recent times, inhibition by caffeine has been ascribed to an effect on calcium stores, but given the high concentrations of caffeine required in all types of studies, the effect of caffeine alone might be a consequence of interference with a number of targets.

We have no satisfactory explanation for the desensitization of neutrophils upon preincubation with ryanodine. Ryanodine is not chemically inactivated by cellular constituents, because application of ryanodine after preincubation with ryanodine does not restore the inhibitory effect. The Ca^{2+} stores for fMLP and ryanodine are apparently different, as was shown in the experiment where the cells were preincubated with caffeine. Though it seems likely that the ryanodine-sensitive Ca^{2+} store is involved in the inhibitory effect of ryanodine on migration, some phenomena, such as the complete loss of inhibitory potency after preincubation with ryanodine while the calcium response remains, require further investigation.

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