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Effects of khellin on contractile responses and $^{45}Ca^{2+}$ movements in rat isolated aorta

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Abstract—The effects of khellin on contractile responses and $^{45}Ca^{2+}$ flux have been studied in rat isolated aortae. Khellin (10^{-5}–$3{\cdot}2 \times 10^{-4}$ M) produced a concentration-dependent inhibition of noradrenaline (10^{-6} M) and high K^+ (80 mM)-induced contractions. At $3{\cdot}2 \times 10^{-4}$ M, khellin increased cAMP levels and reduced $^{45}Ca^{2+}$ influx in resting tissues and in tissues stimulated by noradrenaline (10^{-5} M) and high K^+ without affecting basal $^{45}Ca^{2+}$ efflux or noradrenaline induced $^{45}Ca^{2+}$ efflux. It is concluded that in rat isolated aorta, khellin caused a non-specific inhibition of Ca^{2+} influx but may also exhibit intracellular actions, thus decreasing the availability of Ca^{2+} required for activation. One or more of these mechanisms may be related to an increase in intracellular cAMP levels.

The mechanism responsible for the vasodilator and spasmolytic actions of khellin, the active principle of *Ammi visnaga* L. (Lam.), is not known. Results in isolated smooth muscle suggest that khellin interferes with the chain of events leading from receptor occupation by the stimulatory agent to the effect. Thus, in rat aorta, khellin inhibited the contractile responses induced by caffeine and noradrenaline in Ca-free medium (Ubeda & Villar 1989) and in K^+-depolarized guinea-pig ileum it completely shifted the dose-response curve to Ca^{2+} (Simonis et al 1971; Labrid et al 1977; Hemavathi et al 1979). These results suggest that the effects of khellin may be attributed to an inhibition of Ca^{2+} entry and/or to an intracellular effect, thus decreasing the availability of Ca^{2+} required for smooth muscle activation. The present study was undertaken to investigate the effects of khellin on contractile responses and $^{45}Ca^{2+}$ movements in rat isolated aorta.

Materials and methods

Experimental procedure. Wistar rats of either sex, 200–300 g, were stunned and exsanguinated. The descending thoracic aorta was rapidly excised, cleaned and cut into helical strips as described by Furchgott & Bhadrakom (1953). Strips ($0{\cdot}3 \times 3$ cm) were mounted in 30 mL organ baths containing physiological saline solution (PSS) of the following composition (mM): NaCl 122, KCl 5·9, $CaCl_2$ 1·25, $MgCl_2$ 1·25, $NaHCO_3$ 15 and glucose 11. The solution was bubbled with 95%O_2–5%CO_2 and maintained at 37°C. Contractile responses were measured isometrically by a force-displacement transducer Grass FT03 coupled to a Grass polygraph. The strips were equilibrated for 90–120 min under 1 g of tension before experiments were started.

Following equilibration, aortic strips were contracted with noradrenaline (NA, 10^{-6} M) and high K^+ (80 mM). Control contractile responses for each agonist were obtained at the beginning of the experiment at 30 min intervals until two successive responses were almost identical. The strips were then exposed to the desired concentration of khellin for 10 min, the agonist was re-added to the bath and the contractile response recorded. Only one agonist was used in each experiment. The values of the contractile responses were expressed as percentage of control contraction in each experiment.

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Assay of cAMP. To study the effects on cAMP, aortic strips were equilibrated for 60 min in PSS and then exposed to $3{\cdot}2 \times 10^{-4}$ M of khellin for 10 min. The tissues were immediately frozen with liquid nitrogen, weighed and homogenized in 6% trichloroacetic acid. The levels of cAMP in the extract were measured using a radioimmunoassay kit (RIANEN cAMP [^{125}I]). cAMP levels were expressed as pmol mg^{-1} tissue wet weight.

Measurements of $^{45}Ca^{2+}$ influx and $^{45}Ca^{2+}$ efflux. Rings of rat thoracic aorta (2–3 mm) were equilibrated for 2 h in tubes containing physiological solution (PS) of the following composition (mM): NaCl 140, KCl 4·6, $CaCl_2$ 1·5, $MgCl_2$ 1, Hepes 5 and glucose 10. The solution was adjusted to pH 7·2 and bubbled with 100% O_2. After equilibration experimental rings were treated with khellin for 10 min and control rings with an equivalent amount of solvent (i.e. ethanol). The rings were exposed for 120 s to ^{45}Ca-labelled solution (specific activity 4 μCi mL^{-1}). The amount of Ca^{2+} entering the tissue during such short periods can be assumed to be primarily due to Ca^{2+} influx (Meisheri et al 1981). In some experiments NA (10^{-5} M) and high K^+ (80 mM) were added simultaneously with $^{45}Ca^{2+}$ and only one agonist was tested in each experiment. At the end of a 120 s exposure to the stimulatory agent, the tissues were bathed in ice-cold Ca-free physiological solution containing 2 mM EGTA for 40 min to remove extracellular Ca^{2+} (Meisheri et al 1980, 1981). After this, tissues were removed, blotted, weighed and digested with Soluene overnight at 50°C. Radioactivity was determined in a liquid scintillation counter (LKB Wallac 1211 Rack) as described by Barrigon et al (1984).

To determine $^{45}Ca^{2+}$ efflux, aortic strips were incubated in ^{45}Ca-labelled PS (specific activity 4 μCi mL^{-1}) for 2 h and, after soaking (30 s) in cold non-radioactive solution with 2 mM EGTA, were placed in successive tubes containing 2 mL of PS containing 2 mM EGTA every 5 min for the 90 min duration of the washout. It has been demonstrated that $^{45}Ca^{2+}$ efflux is much more distinctive in Ca-EGTA medium compared with Ca-free solution (Deth & Van Breemen 1977). In the last 8 tubes of the washout, khellin was added. Radioactivity lost into the tubes and present in the tissues at the end of the experiment was measured as described for influx experiments. The data obtained were plotted as desaturation curves which illustrated the decline of tissue $^{45}Ca^{2+}$ content with time.

Noradrenaline-dependent $^{45}Ca^{2+}$ efflux was estimated as described by Godfraind (1976). Aortic strips were preincubated for 2 h in [^{45}Ca]-PSS (specific activity 2 μCi mL^{-1}) and khellin was added during the last 10 min in the experimental strips. Tissues were then rinsed for 5 min in non-radioactive Ca-free solution containing 0·2 mM EGTA before being transferred to non-radioactive Ca-free solution also containing EGTA and NA ($2{\cdot}5 \times 10^{-5}$ M) for a further 2 min. The strips were then placed for 5 min in ice-cold lanthanum-containing solution (composition mM: NaCl 136, KCl 4·6, $MgCl_2$ 1, $LaCl_3$ 50, glucose 11 and Tris maleate 6, pH 7·2) to remove extracellular

Ca^{2+} from the tissue. Khellin was present during this procedure in the experimental preparations; control preparations were rinsed in non-radioactive Ca-free solution for 7 min. Radioactivity present in the tissues at the end of the experiment was determined as described for influx experiments.

Drugs. The drugs and chemicals used were: khellin (Sarsynthèse), noradrenaline bitartrate (Sigma), potassium chloride (Merck) and calcium chloride (Merck). $^{45}Ca^{2+}$ (sp. act. 2·02 mCi mL^{-1}) was purchased from the Radiochemical Centre, Amersham). Khellin was initially dissolved in ethanol to prepare a 10^{-2} M stock solution and dilutions made in physiological solution. The final ethanol concentrations did not significantly affect the results. Ascorbic acid (10^{-4} M) was added to each solution of noradrenaline, made up fresh daily.

Statistics. The results are expressed as mean ± s.e.m. Statistical significance was evaluated by Student's *t*-test for paired or unpaired data and differences were considered significant for $P<0·05$.

Results

Effects on NA- and high K^+- induced contractions. Fig. 1 shows the time course of the inhibitory effects of khellin, 10^{-5}–$3·2\times10^{-4}$ M, on contractile responses induced by (A) NA (10^{-6} M) and (B) high K^+ (80 mM). Khellin produced a concentration-dependent decrease of both phasic and tonic components of NA-induced contractions, which reached significant values ($P<0·05$) at concentrations higher than 10^{-5} M. Furthermore, at all concentrations tested khellin also produced a significant inhibitory effect on high K^+-induced contractions ($P<0·05$). However, the tonic component of NA-induced contractions appeared to be more sensitive to the inhibitory effect of khellin than that of high K^+-induced contractions. The inhibitory effect of khellin was reversed by washing with drug-free physiological solution.

Effects of khellin on cAMP levels. In aortic strips pretreated with $3·2\times10^{-4}$ M, khellin significantly increased the levels of cAMP from 0·060 ± 0·010 to 0·111 ± 0·011 pmol mg^{-1} wet weight ($P<0·05$).

Effects of khellin on $^{45}Ca^{2+}$ influx and $^{45}Ca^{2+}$ efflux. From the previous experiments two concentrations (1·6 and $3·2\times10^{-4}$ M) were selected to study the effects of khellin on $^{45}Ca^{2+}$ fluxes. Fig. 2 shows the effects of khellin on $^{45}Ca^{2+}$ influx in resting, unstimulated, aortic rings and in rings previously stimulated by

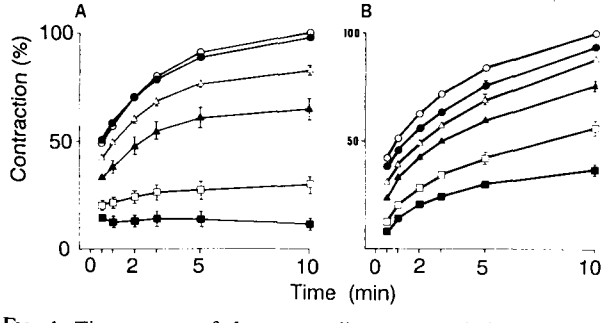

FIG. 1. Time course of the contractile responses induced by (A) noradrenaline (10^{-6} M) and (B) high K^+ (80 mM) in rat isolated aortic strips. Khellin was added 10 min before the addition of the agonists. Each point represents the mean of 6 experiments; vertical bars show the s.e.m. ○ Controls. ● Khellin, 10^{-5} M, △ 4×10^{-5} M, ▲ 8×10^{-5} M, □ $1·6\times10^{-4}$ M, ■ $3·2\times10^{-4}$ M.

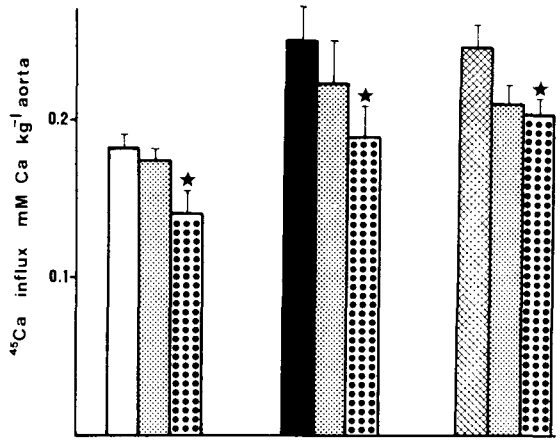

FIG. 2. Effects of khellin, $1·6\times10^{-4}$ M (stippled columns) and $3·2\times10^{-4}$ M (dotted columns) on $^{45}Ca^{2+}$ influx in resting rings (open column) and in rings stimulated by NA (10^{-5} M, solid column) or high K^+ (80 mM, hatched column). Values are the mean of 7–8 experiments; vertical lines represent the s.e.m. $^*P<0·05$.

10^{-5} M NA and 80 mM K^+. In eight resting aortae, khellin at $1·6\times10^{-4}$ M had no effect while at $3·2\times10^{-4}$ M it reduced $^{45}Ca^{2+}$ content ($P<0·05$). Addition of NA and high K^+ increased $^{45}Ca^{2+}$ content over control values ($P<0·05$). At $1·6\times10^{-4}$ M, khellin had *no significant effects either on* $^{45}Ca^{2+}$ *influx* stimulated by NA or higher K^+, whereas at $3·2\times10^{-4}$ M khellin significantly decreased $^{45}Ca^{2+}$ influx stimulated both by NA ($n=7$, $P<0·05$) or high K^+ ($n=7$, $P<0·05$).

The effects of khellin, $3·2\times10^{-4}$ M, on $^{45}Ca^{2+}$ efflux were studied in resting, unstimulated aortic strips. The strips were washed in physiological solution containing EGTA during the first 50 min of the 90 min washout and with the same solution containing khellin for the final 40 min. The analysis of the desaturation curves (Fig. 3) showed that at this concentration khellin had no effect on the slopes of the regression lines of the slow component (last 40 min) of the washout. Thus, the values of the slopes in non-treated strips was −0·025 ± 0·001 compared with −0·026 ± 0·002 in khellin-treated strips ($n=4$, $P>0·05$). This result suggested that khellin had no effect on $^{45}Ca^{2+}$ efflux.

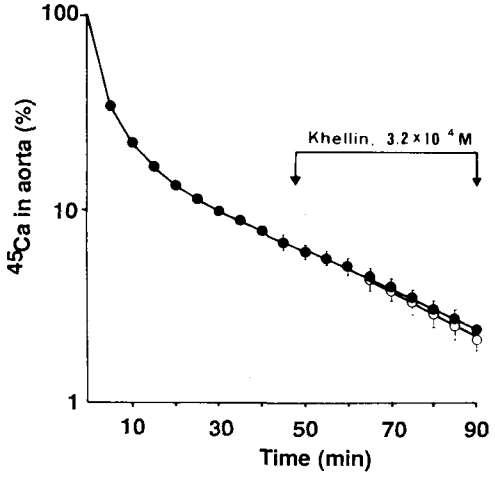

FIG. 3. Effects of khellin on percentage of $^{45}Ca^{2+}$ remaining in the aorta. Arrows indicated the interval of exposure to khellin during the washout. Each point represents the mean ± s.e.m. of 4 experiments. ● Controls. ○ Khellin, $3·2\times10^{-4}$ M.

The effects of khellin were also studied on NA-induced $^{45}Ca^{2+}$ efflux. The addition of NA ($2{\cdot}5 \times 10^{-5}$ M) caused an increase in $^{45}Ca^{2+}$ efflux, reducing tissue content of exchangeable Ca^{2+} from $1{\cdot}784 \pm 0{\cdot}196$ to $1{\cdot}218 \pm 0{\cdot}126$ mM kg^{-1} wet weight ($n = 6$, $P < 0{\cdot}05$). However, at $3{\cdot}2 \times 10^{-4}$ M, khellin had no effect on NA-stimulated $^{45}Ca^{2+}$ efflux ($1{\cdot}033 \pm 0{\cdot}168$ mM kg^{-1} wet weight, $n = 5$, $P > 0{\cdot}05$).

Discussion

The present study demonstrates that in rat isolated aorta, khellin, a furanochromone with spasmolytic and vasodilator properties (Anrep et al 1949), inhibits the contractile responses induced by NA and high K^+ and decreases $^{45}Ca^{2+}$ influx in resting tissues as well as in tissues stimulated by NA and high K^+.

In rat isolated aorta the NA- and high K^+- induced contractions have been resolved into phasic and tonic components (Godfraind & Kaba 1972; Van Breemen et al 1979). It is generally accepted that Ca^{2+} enters vascular smooth muscle cells through voltage-operated channels stimulated by depolarization and through receptor-operated channels stimulated by a specific agonist (Bolton 1979). An increase in Ca^{2+} influx through receptor-operated channels seems to determine the tonic component of NA-induced responses (Steinsland et al 1973; Deth & Van Breemen 1974; Godfraind 1976), whereas the high K^+-induced contractile response is thought to be the result of an increased Ca^{2+} influx through voltage-sensitive calcium channels (Bolton 1979).

Khellin inhibited the contractile responses induced by high K^+ and the tonic component of NA-induced responses, which suggested that it may inhibit Ca^{2+} entry through both voltage- and receptor-operated channels of the vascular smooth muscle membrane. In the present experiments, khellin inhibited to a similar extent $^{45}Ca^{2+}$ influx in aortae stimulated by high K^+ and NA. Thus, it appears to act as a non-specific inhibitor of $^{45}Ca^{2+}$ influx, an effect which may explain the decrease in cardiac contractility reported in animals (Helmy 1963) and in man (Jordan 1958).

Khellin also inhibited the phasic component of NA-induced contractions which has been attributed to the release of Ca^{2+} from intracellular stores (Godfraind & Kaba 1972; Deth & Van Breemen 1977). This result suggests that khellin may also inhibit the release of Ca^{2+} from an agonist releasable store. The stimulation of efflux rate by NA is thought to reflect the release of Ca^{2+} from an intracellular store (Deth & Van Breemen 1977). However, since khellin had no effect on resting or NA-stimulating $^{45}Ca^{2+}$ efflux, the inhibition of the phasic component of NA-induced contractions did not seem to be related to a decrease in the release of Ca^{2+} from an intracellular store and/or to an enhanced extrusion of Ca^{2+} from the vascular smooth muscle. The observed inhibitory effect of khellin can probably be explained by its ability to increase intracellular cAMP levels, an effect which has been demonstrated in the present experiments.

In conclusion, the data from the present study show that in rat isolated aorta, khellin produces a non-specific inhibition of smooth muscle contractility by acting probably at multiple sites of action to decrease the availability of Ca^{2+} required for smooth muscle activation.

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References

Anrep, G. V., Kenawy, M. R., Barsoum, G. S. (1949) The coronary vasodilator action of khellin. Am. Heart J. 37: 531–542

Barrigon, S., Tejerina, T., Delgado, C., Tamargo, J. (1984) Effects of chlorbutol on ^{45}Ca movements and contractile responses of rat aorta and its relevance to the actions of syntocinon. J. Pharm. Pharmacol. 36:521–526

Bolton, T. B. (1979) Mechanism of action of transmitters and other substances on smooth muscle. Physiol. Rev. 59: 606–718

Deth, R., Van Breemen, C. (1974) Relative contribution of Ca^{2+} influx and cellular Ca^{2+} release during induced activation of rabbit aorta. Pflugers Arch. 348: 13–22

Deth, R., Van Breemen, C. (1977) Agonist induced release of intracellular Ca in the rabbit aorta. J. Membrane Biol. 30: 363–380

Furchgott, R. F., Bhadrakom, S. (1953) Reactions of strips of rabbit aorta to epinephrine, isopropylarterenol, sodium nitrate and other drugs. J. Pharmacol. Exp. Ther. 108: 124–143

Godfraind, T. (1976) Calcium exchange in vascular smooth muscle, action of noradrenaline and lanthanum. J. Physiol. 260: 21–35

Godfraind, T., Kaba, A. (1972) The role of calcium in the action of drugs on vascular smooth muscle. Arch. Intern. Pharmacodyn. 196: 35–49

Helmy, R. (1963) Comparative study of the effects of some coronary dilators on cardiac volume. J. Egypt. Med. Ass. 46: 833–842

Hemavathi, K. G., Bhatt, J. D., Amin, D. V., Gulati, O. D., Shah, D. S. (1979) Antagonism of calcium-induced contractions by some nonspecific spasmolytics in depolarized smooth muscles. Indian J. Physiol. Pharmacol. 23: 261–268

Jordan, H. (1958) Cardiovasculare wirkungen intravenoser khellin-injektionen. Arzneim. Forsch. 8: 141–143

Labrid, C., Dureng, G., Bert, H., Duchene-Marullaz, P. (1977) Nonspecific antispasmodics and barium induced contraction. J. Pharmacol. 8: 169–184

Meisheri, K. D., Palmer, R. F., Van Breemen, C. (1980) The effects of amrinone on contractility, Ca^{2+} uptake and cAMP in smooth muscle. Eur. J. Pharmacol. 61: 159–165

Meisheri, K., Hwang, O., Van Breemen, C. (1981) Evidence for two separate Ca^{2+} pathways in smooth muscle plasmalemma. J. Membr. Biol. 59: 19–25

Simonis, A. M., Ariëns, E. J., Van den Broeke, J. J. W (1971) Non-competitive spasmolytics as antagonists of Ca^{++}-induced smooth muscle contraction. J. Pharm. Pharmacol. 23: 107–110

Steinsland, O., Furchgott, R. F., Kirpekar, S. (1973) Biphasic vasoconstriction of the rabbit ear artery. Circ. Res. 32: 49–58

Ubeda, A., Villar, A. (1989) Relaxant actions of khellin on vascular smooth muscle. J. Pharm. Pharmacol. 41: 236–241

Van Breemen, C., Aaronson, P., Loutzenhinser, R. (1979) Na-Ca interactions in mammalian smooth muscle. Pharmacol. Rev. 30: 167–208