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Effects of Bay K 8644 on ^{45}Ca uptake and efflux and on contraction in the rabbit aorta

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1 Contractions induced by K^+, noradrenaline and 11,9-epoxymethano prostaglandin H_2 (11,9-epoxymethano PGH_2) were accompanied by a large, moderate and negligible stimulation of ^{45}Ca uptake in rabbit aortic rings, respectively. Bay K 8644, 14 and 56 nM, enhanced both the contraction and the ^{45}Ca uptake stimulated by all 3 agonists.

2 In the absence of agonists, Bay K 8644 (14 and 56 nM) caused a small contraction and increase in ^{45}Ca uptake.

3 ^{45}Ca efflux was increased by noradrenaline, and Bay K 8644 augmented this.

4 In Ca-free solution, contractions induced by noradrenaline or 11,9-epoxymethano PGH_2 were not augmented by Bay K 8644.

5 Nifedipine (0.1 μM) antagonized ^{45}Ca uptake stimulated by K^+ or noradrenaline. Nifedipine also reduced the stimulant effect of Bay K 8644 on ^{45}Ca uptake in the presence of all three agonists.

6 It is concluded that, in the rabbit aorta, Bay K 8644 enhances the opening of Ca channels both during depolarization and in the presence of receptor-specific agonists and is also able to open Ca channels under basal conditions. Bay K 8644 appears not to reduce Ca efflux or enhance Ca release from intracellular stores.

Introduction

Bay K 8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate), a novel 1,4-dihydropyridine derivative, is an analogue of nifedipine. It has been reported to have positive inotropic and vasoconstrictor effects, opposite to those of nifedipine, and to enhance K^+-induced, but not noradrenaline-induced contractions of the rabbit aorta (Schramm *et al.*, 1983). An attractive interpretation of these results is that Bay K 8644 facilitates, instead of blocking, calcium channels (Schramm *et al.*, 1983) and patch-clamp studies in cardiac muscle support this idea (Brown *et al.*, 1984; Hess *et al.*, 1984). However, it is also possible that Bay K 8644 could augment contraction by inhibiting Ca efflux or sequestration, and we have therefore investigated the effects of Bay K 8644 on ^{45}Ca uptake and ^{45}Ca efflux in the rabbit aorta. We have also studied its interaction with the agonists K^+, noradrenaline and the thromboxane mimetic, 11,9-epoxymethano prostaglandin H_2 (11,9-epoxymethano PGH_2), which have distinct effects on excitation-contraction coupling.

Methods

Tissue preparation

Rabbits weighing 2.0–2.5 kg were killed by a blow to the neck. The thoracic aorta was rapidly removed, transferred to a dissection bath containing HEPES-Krebs solution (composition mM: NaCl 144, KCl 5.8, $MgCl_2$ 1.2, HEPES 5.0, $CaCl_2$ 2.5 and glucose 11.1, pH 7.3) bubbled with O_2, or Krebs-Henseleit solution (composition, mM: NaCl 118, $NaHCO_3$ 25, KCl 4.6, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5 and glucose 11.1) aerated with 95% O_2 and 5% CO_2, and kept at 37°C. The aorta was cleared of adherent tissues and cross-sectioned into rings.

^{45}Ca uptake

^{45}Ca uptake in rabbit aorta was conducted using a modified 'Lanthanum method', where a low temperature (0.5°C) and incubation with a high La^{3+} concentration (50 mM) was chosen (Godfraind, 1976;

Deth, 1978; Hay & Wadsworth, 1984). Preparations were incubated for about 1 h in 2 ml of HEPES-Krebs solution, then transferred into 3 ml of HEPES-Krebs solution containing Bay K 8644 or/and nifedipine. After 25 min, the tissues were transferred to 1 ml of HEPES-Krebs solution containing ^{45}Ca (0.5 μCi ml^{-1}), and Bay K 8644 or/and nifedipine, as appropriate, for 5 min. Agonists were then added 5 min before the tissues were finally placed in 3 ml of calcium-free HEPES-Krebs solution containing $LaCl_3$ 50 mM for 60 min. All the incubations were carried out at 37°C, apart from lanthanum treatment which was done at 0.5°C. For control preparations Bay K 8644, nifedipine or/and agonists were omitted. Following extraction of the tissues overnight with 5.4 ml of disodium edetate (EDTA) (5 mM), a scintillation mixture (Scintran Cocktail T, BDH Chemical Ltd.) was added. The radioactivity present was counted in a Packard Tri-Carb 460 CD scintillation counter and the cellular ^{45}Ca content calculated.

^{45}Ca efflux

^{45}Ca efflux was measured by a superfusion method using paired preparations which were run simultaneously. After 1 h loading with ^{45}Ca (2 μCi ml^{-1}) in 1 ml of HEPES-Krebs solution, the preparations were suspended under 1 g tension and superfused with HEPES-Krebs solution with or without Bay K 8644 at a rate of 2.7 ml min^{-1} for 60–80 min. Agonists were added and superfused for 20 min. The superfusate was collected every 2 min, and at the end of superfusion tissues were extracted with 5.4 ml of EDTA (5 mM) overnight. Scintillation mixture was then added to the superfusate and tissue extracts, and the radioactivity was counted in a Packard Tri-Carb 460 CD scintilla-

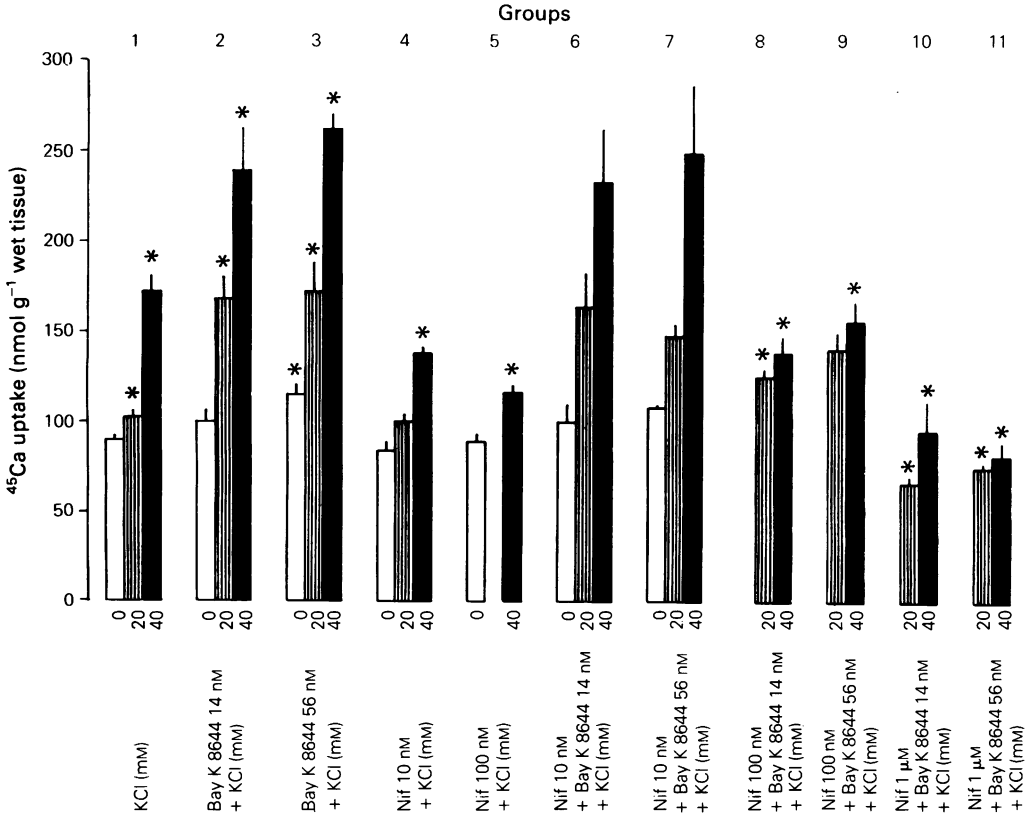

Figure 1 Rabbit aorta: effects of Bay K 8644 and nifedipine (Nif) on KCl-induced cellular ^{45}Ca uptake. The columns show the mean with ± s.e.mean indicated by vertical lines, n = 5–14. *Significantly different from appropriate control (KCl and Bay K 8644 compared with basal; appropriate columns in groups 2, 3, 4 and 5 compared with group 1; groups 6, 8, 10 compared with group 2; groups 7, 9, 11 compared with group 3).

tion counter and expressed as rate coefficient of efflux (Weiss, 1977). Tissues were aerated with O_2 and kept at 37°C during ^{45}Ca loading and efflux. The superfusate in all efflux experiments contained EDTA (10 μM) and ascorbic acid (20 μM) to reduce degradation of noradrenaline (Bell *et al.*, 1984).

Tension experiments

Rabbit aorta rings were suspended under 2–3 g resting tension in 15 ml of Krebs-Henseleit solution aerated with 95% O_2 and 5% CO_2 and kept at 37°C. In calcium-free experiments $CaCl_2$ was omitted; when a chelator was added, this is stated in Results. Isometric tension was measured with a Grass force displacement transducer (FTO3D) and recorded on a Grass Polygraph (Model 79D). Each preparation was allowed 1 h to stabilize.

Drugs and chemicals

The following drugs were used: nifedipine, Bay K 8644 (both donated by Bayer), 11,9-epoxymethano PGH_2 (donated by Upjohn Co.), noradrenaline (Sigma), ethylenediaminetetraacetic acid disodium (EDTA) (Sigma) and $^{45}CaCl_2$ (Amersham).

All the experiments were conducted in the dark because of the photosensitivity of nifedipine and Bay K 8644.

Statistics

Results are expressed as means ± s.e.mean. Statistical analysis of the data was made using Student's *t* test and the 0.05 level of probability was regarded as significant.

Results

^{45}Ca uptake

La^{3+}-resistant ^{45}Ca uptake was induced by KCl, noradrenaline and 11,9-epoxymethano PGH_2, and two concentrations were chosen for each drug (producing about 50% and about 80% of the maximum contraction).

Potassium In the absence of agonist, basal ^{45}Ca uptake was 89 ± 3.0 nmol g^{-1} using a 10 min uptake period under the conditions of these experiments. KCl (20 and 40 mM) increased ^{45}Ca uptake, the higher concentration approximately doubling the basal uptake (Figure 1, first group of columns).

Bay K 8644 (56 nM) caused a small increase in ^{45}Ca uptake and also potentiated the effect of KCl, which was increased approximately 3 fold with KCl (20 mM) and nearly 2 fold with KCl (40 mM). The potentiating effect of Bay K 8644 seemed to be maximal at the lower concentration (14 nM) (Figure 1, Groups 2 and 3).

Nifedipine (0.01 and 0.1 μM) had no effect on basal ^{45}Ca uptake but antagonized KCl (by 33% at 0.01 μM and by 64% at 0.1 μM; Figure 1, groups 4 and 5). The augmenting effect of Bay K 8644 was reduced by nifedipine (0.1 μM) by about two thirds, i.e. nifedipine had a similar effect on KCl-induced uptake in the presence and in the absence of Bay K 8644 (groups 8–9). The lower concentration of nifedipine (0.01 μM) had little effect on Bay K 8644-potentiated-KCl-induced ^{45}Ca uptake (Figure 1, groups 6–7). A higher concentration of nifedipine (1 μM) had a very marked effect on ^{45}Ca uptake in the combined presence of KCl and Bay K 8644. However, this is difficult to interpret as the values for agonist-stimulated ^{45}Ca uptake were below basal, indicating that nifedipine at this concentration had itself reduced ^{45}Ca uptake (Figure 1, last 2 groups of columns).

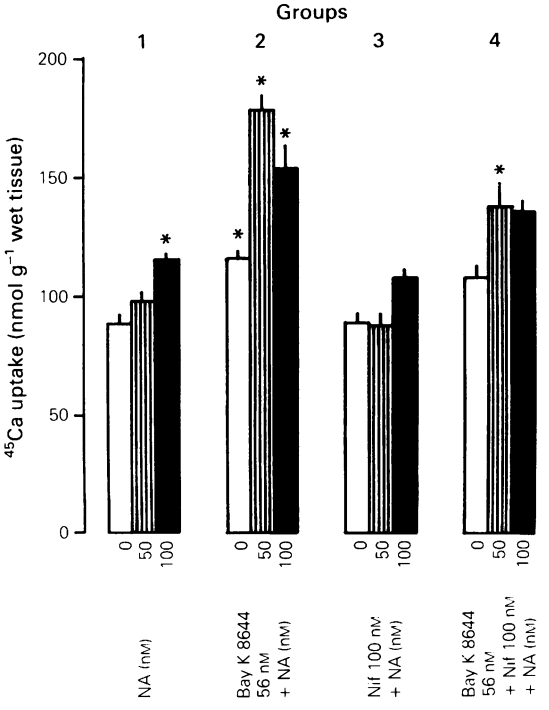

Figure 2 Rabbit aorta: effects of Bay K 8644 and nifedipine (Nif) on noradrenaline (NA)-induced cellular ^{45}Ca uptake. The columns show the mean with ± s.e.mean shown by vertical lines, $n = 5–14$. *Significantly different from appropriate control (NA and Bay K 8644 compared with basal; appropriate columns in groups 2 and 3 compared with group 1; group 4 compared with 2).

Noradrenaline As shown in Figure 2, noradrenaline caused a small, probably dose-dependent increase in ^{45}Ca uptake in rabbit aorta. Bay K 8644 at 56 nM potentiated noradrenaline-induced ^{45}Ca-uptake. Nifedipine at 100 nM produced a small inhibition of noradrenaline-induced ^{45}Ca uptake and inhibited Bay K 8644-potentiated noradrenaline-induced ^{45}Ca uptake, at least at a noradrenaline concentration of 50 nM.

11,9-Epoxymethano PGH_2 Figure 3 shows that 11,9-epoxymethano PGH_2 had either no, or very little effect on the basal ^{45}Ca uptake in the rabbit aorta. However in the presence of Bay K 8644, the ^{45}Ca uptake induced by 11,9-epoxymethano PGH_2 was greatly increased. Nifedipine at 0.1 μM had no significant effect on ^{45}Ca uptake in the presence of 11,9-epoxymethano PGH_2, but significantly reduced Bay K 8644-potentiated 11,9-epoxymethano PGH_2-induced ^{45}Ca uptake.

^{45}Ca efflux

Two different types of HEPES buffered superfusates were used: one contained normal (2.5 mM) Ca and the other was Ca-free (with EDTA 10 μM) ($n = 3$ for each superfusate). The results of typical experiments are shown in Figure 4.

Noradrenaline at 500 nM markedly increased ^{45}Ca efflux with 2.5 mM Ca superfusate and this was greatly potentiated by Bay K 8644 (56 nM) (Figure 4a). Bay K 8644 (56 nM) alone produced a small increase in ^{45}Ca efflux rate. With Ca-free HEPES-Krebs solution the efflux curve was steeper and ^{45}Ca remaining in the tissue at the end of experiment was higher, indicating a reduced basal ^{45}Ca efflux rate. In the Ca-free solution, noradrenaline alone did not affect the efflux rate, but it produced an increase in ^{45}Ca efflux rate in the presence of Bay K 8644 (56 nM) (Figure 4b).

Tension experiments

Bay K 8644 at 56 nM produced a small contraction (27 ± 8.5%, $n = 3$ of the contraction produced by noradrenaline 50 nM) (Figure 5). Bay K 8644 also potentiated the contractile responses to noradrenaline, 11,9-epoxymethano PGH_2 (Figure 5) and KCl. In the presence of Bay K 8644 (56 nM), the contraction produced by noradrenaline was 360 ± 36% ($n = 3$) of that produced by noradrenaline alone. Bay K 8644 potentiated responses to 11,9-epoxymethano PGH_2 in a Bay K 8644-preincubated preparation as well as in a preparation where tension had developed to 11,9-epoxymethano PGH_2. However, when Bay K 8644 was added to a preparation contracted by noradrenaline only a small increase in tone could be elicited.

In nominally Ca-free solution, potentiating effects of Bay K 8644 on responses to noradrenaline and 11,9-epoxymethano PGH_2 remained. However, when preparations were pre-incubated with the Ca-free solution containing EDTA (10^{-5} M) for one hour, the potentiating effect of Bay K 8644 on the response to noradrenaline or 11,9-epoxymethano PGH_2 was abolished (Figure 6).

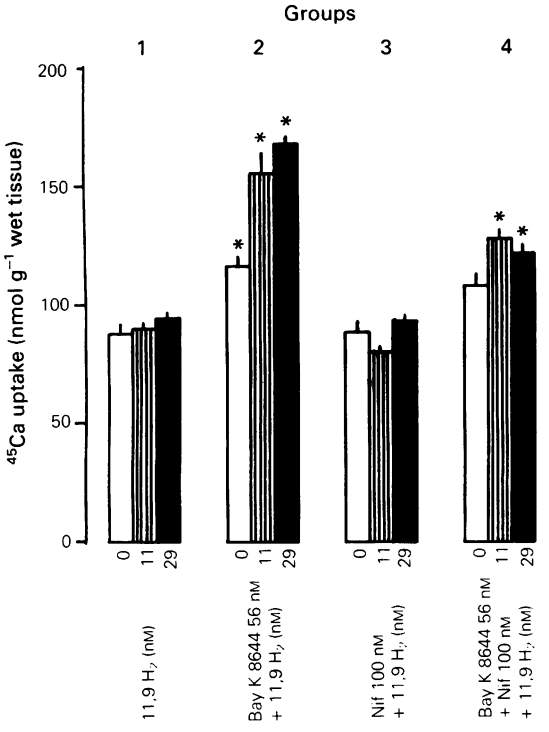

Figure 3 Rabbit aorta: effects of Bay K 8644 and nifedipine (Nif) on 11,9-epoxymethano PGH_2 (11,9H_2)-induced ^{45}Ca uptake. The columns show the mean with ± s.e.mean indicated by vertical lines, $n = 4-14$. *Significantly different from appropriate control (11,9 H_2 and Bay K 8644 compared with basal; appropriate columns in groups 2 and 3 compared with group 1; group 4 compared with group 2).

Discussion

Potassium causes contraction of smooth muscle primarily by opening voltage-dependent Ca channels and admitting extracellular Ca^{2+} into the cell. In agreement with other workers (van Breemen *et al.*, 1972; Karaki *et al.*, 1984), our results show that potassium dose-dependently increases cellular ^{45}Ca uptake, an effect prevented by nifedipine, a calcium channel inhibitor. It has been reported that vasoconstriction induced by activation of α_2-adrenoceptors requires extracellular Ca, while vasoconstriction

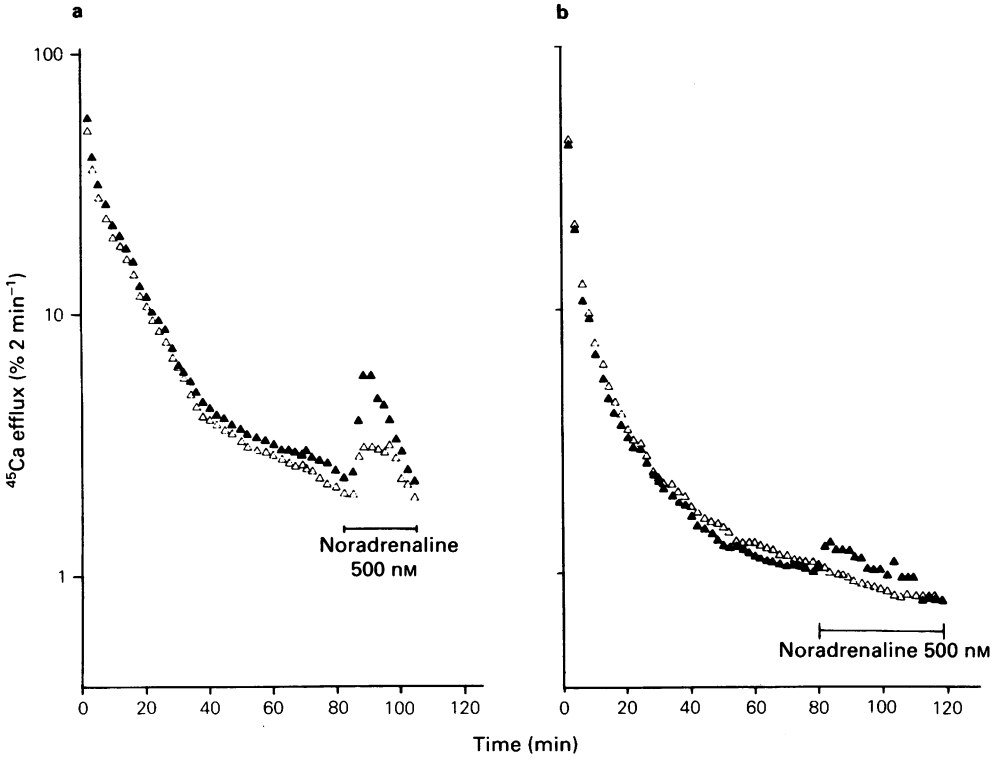

Figure 4 Rabbit aorta: effects of Bay K 8644 on noradrenaline-induced Ca efflux using (a) a superfusing solution containing 2.5 mM Ca and (b) a Ca-free superfusing solution. Two preparations were run simultaneously. After superfusion for 80 min with HEPES-Krebs solution containing Bay K 8644 (56 nM) (▲) or without Bay K 8644 (△), noradrenaline (500 nM) was added for the period indicated by the horizontal bar. The rate of Ca efflux is expressed as the percentage of the exchangeable Ca present that is released in each 2 min sampling period.

evoked by stimulation of α_1-adrenoceptors is caused by releasing Ca from intracellular stores (van Zwieten *et al.*, 1983). However, other workers (Cauvin *et al.*, 1982) have shown that in rabbit aorta, α_1-adrenoceptor stimulation induced both Ca influx and intracellular Ca release. The results in this study confirm that noradrenaline increases ^{45}Ca uptake, but this is smaller than the effect of potassium. In our experiments, noradrenaline-induced ^{45}Ca uptake was slightly reduced by nifedipine (0.1 μM). 11,9-epoxymethano PGH_2 is believed to cause contraction primarily by mobilising Ca from intracellular stores and our experiments confirm that it has negligible effects on ^{45}Ca uptake at concentrations that give submaximal contractions. However, in high concentrations, Ca uptake is also increased (Loutzenhiser & van Breeman, 1981). Thus the three vasoconstrictors, potassium, noradrenaline and 11,9-epoxymethano PGH_2 cause contractions of the rabbit aorta that are (respectively) wholly dependent, partly dependent and largely independent of Ca influx. Bay K 8644 augmented contractions produced by each of these 3 vasoconstrictors.

Potassium- and noradrenaline-stimulated ^{45}Ca uptake was augmented by Bay K 8644. Furthermore, in the presence of Bay K 8644, 11,9-epoxymethano PGH_2 caused a distinct rise in ^{45}Ca uptake. These results suggest that Bay K 8644 is facilitating the opening of Ca channels which is in agreement with the augmentation of inward Ca currents in ventricular muscle (Hess *et al.*,1984; Brown *et al.*, 1984). Since high potassium opens voltage-dependent channels while receptor-specific agonists open receptor-operated channels (Bolton, 1979), our results could indicate that Bay K 8644 has a similar effect on both types of Ca channel. Our results are not in agreement with the conclusion of Schramm *et al.* (1983) that Bay K 8644 acts specifically on voltage-dependent channels, unless it is additionally supposed that noradrenaline (and, under suitable conditions, 11,9-

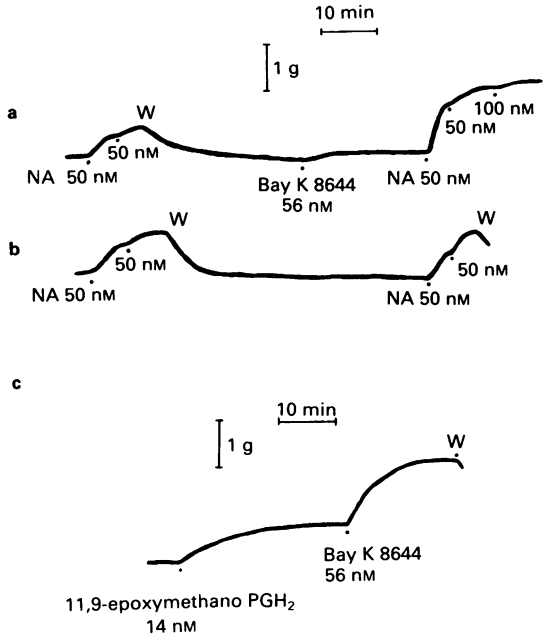

Figure 5 Rabbit aorta: contraction produced by Bay K 8644 and receptor-specific agonists. (a) Bay K 8644 produced a contraction on its own and augmented the contractions produced by noradrenaline (NA). (b) Noradrenaline contractions are reproducible in the control preparation. (c) Bay K 8644 augments the contraction produced by 11,9 epoxymethano PGH_2. W indicates wash.

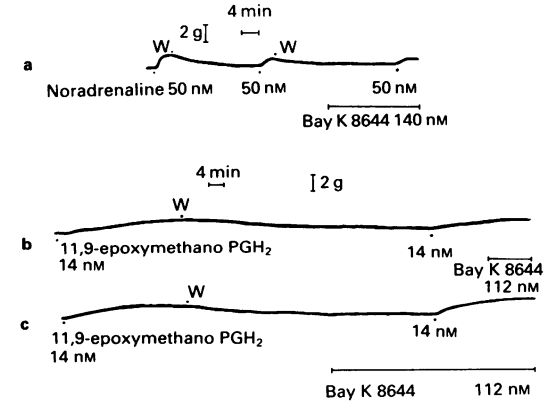

Figure 6 Rabbit aorta: effects of Bay K 8644 and receptor-specific agonists on tension in Ca-free Krebs solution containing EDTA (10 μM). Bay K 8644 did not augment contractions produced by (a) noradrenaline or (b and c) 11,9 epoxymethano PGH_2 in calcium-free conditions. Bay K 8644 present for periods indicated by horizontal bars. W indicates wash.

epoxymethano PGH_2) acts by facilitating the opening of voltage-dependent channels.

The increased Ca efflux produced by noradrenaline in a Ca containing solution is partly secondary to the increase in Ca influx. Therefore, since we have shown that noradrenaline-induced Ca uptake is augmented by Bay K 8644, it is to be expected that Ca efflux will be augmented also, and this is what we observed. When the efflux is performed in a Ca-free solution, noradrenaline can no longer induce a $^{40}Ca/^{45}Ca$ exchange and the augmentation of Ca efflux that occurs is due to noradrenaline-induced Ca release from intracellular stores. Bay K 8644 augmented Ca efflux under these conditions. We can rule out an effect of Bay K 8644 on the Ca release process itself, since we found that Bay K 8644 does not augment contraction induced by noradrenaline or 11,9-epoxymethano PGH_2 in Ca-free solution. Thus we conclude that Bay K 8644 enhances the ability of the Ca channels to carry Ca in the outward, as well as in the inward, direction, in agreement with Hess *et al.* (1984) using patch- clamp studies.

Some of our experiments were designed to investigate the possibility that Bay K 8644 might affect Ca extrusion, sequestration or release. Bay K 8644 did not affect contraction due solely to release of intracellular Ca (11,9-epoxymethano PGH_2 or noradrenaline in Ca-free solution), from which we conclude that it does not enhance Ca release or inhibit sequestration. Furthermore, Bay K 8644 did not inhibit ^{45}Ca efflux. Thus the existing data are consistent with the conclusion that the augmentation of contraction in vascular smooth muscle produced by Bay K 8644 is entirely attributable to effects on membrane Ca channels.

In the absence of agonist stimulation, Bay K 8644 caused a small contraction and a small increase in ^{45}Ca uptake. Thus the effect of Bay K 8644 is not just to make the Ca channels more responsive to depolarization or to agonists, but also to open a small proportion of the Ca channels under basal conditions. We should therefore expect Bay K 8644 alone to cause some conductance change.

Nifedipine at 0.01 and 0.1 μM reduced but did not abolish the increase in ^{45}Ca uptake caused by K^+ or noradrenaline. Nifedipine also reduced the augmenting effect of Bay K 8644 in the presence of all 3 agonists. Nifedipine had a quantitatively similar effect against Bay K 8644 and against K^+ alone. From these results, two conclusions are possible. Nifedipine may be competing with Bay K 8644, as is suggested by ligand binding studies (Bellemann *et al.*, 1984). Alternatively, the effect of Bay K 8644 may be fundamentally unaltered by nifedipine so that it is still able to facilitate the opening of those channels that remain unblocked in the presence of nifedipine.

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