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Endogenous cannabinoid anandamide directly inhibits voltage-dependent Ca^{2+} fluxes in rabbit T-tubule membranes

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Abstract

The effect of the endogenous cannabinoid, anandamide on Ca^{2+} flux responses mediated by voltage-dependent Ca^{2+} channels was studied in transverse tubule membrane vesicles from rabbit skeletal muscle. Vesicles were loaded with $^{45}Ca^{2+}$ and membrane potentials were generated by establishing K^+ gradients across the vesicle using the ionophore, valinomycin. Anandamide, in the range of 1–100 μM, inhibited depolarization-induced efflux responses. Anandamide also functionally modulated the effects of nifedipine (1–10 μM) and Bay K 8644 (1 μM) on Ca^{2+} flux responses. Pretreatment with the specific cannabinoid receptor antagonist, SR141716A (1 μM), pertussis toxin (5 μg/ml), the amidohydrolase inhibitor, phenylmethylsulfonyl fluoride (0.2 mM) or the cyclooxygenase inhibitor, indomethacin (5 μM) did not alter the inhibition of efflux responses by anandamide. Arachidonic acid (10–100 μM) also effectively inhibited $^{45}Ca^{2+}$ efflux from membrane vesicles. In radioligand binding studies, it was found that both anandamide and arachidonic acid inhibited the specific binding of [^{3}H]PN 200-110 to transverse tubule membranes with IC_{50} values of 4.4 ± 0.7 and 13.4 ± 3.5 μM, respectively. These results indicate that anandamide, independent of cannabinoid receptor activation, directly inhibits the function of voltage-dependent calcium channels and modulates the specific binding of calcium channel ligands of the dihydropyridine class.

Keywords: Ca^{2+} channel; Anandamide; Skeletal muscle

1. Introduction

In the search for endogenous ligands that modulate the activity of voltage-dependent Ca^{2+} channels (VDCCs), a number of active substances have been identified whose chemical structures vary from novel small peptides to relatively simple chemicals such as ascorbic acid (Janis et al., 1988; Callewaert et al., 1989; Ebersole and Molinoff, 1992). An endogenous lipid soluble substance that modulates the binding of the dihydropyridine class of Ca^{2+} channel antagonists to rat cortex and brain membranes has been isolated and identified as arachidonylethanolamide (Johnson et al., 1993). Interestingly, the same compound was also identified as anandamide, an endogenous ligand for cannabinoid receptors (Devane et al., 1992).

Inhibition of neuronal Ca^{2+} currents by anandamide and cannabinoids has been demonstrated in earlier studies (Caulfield and Brown, 1992; Mackie et al., 1994, 1995; Twitchel et al., 1997). However, there has been no report on the effect of anandamide on the function of VDCCs of skeletal muscle which, in terms of its molecular structure, is the best characterized Ca^{2+} channel subtype (Dunn et al., 1994). Furthermore, since VDCCs are known to be inactivated by rises in intracellular Ca^{2+}, possible secondary effects of anandamide on intracellular Ca^{2+} homeostasis (Martin et al., 1994; Di Marzo and Fontana, 1995) also need to be investigated.

We have previously developed a technique to measure Ca^{2+} fluxes mediated by VDCCs in purified transverse (T)-tubule membranes (Dunn, 1989; Oz et al., 1993). These membranes form sealed, inside-out vesicles that are devoid of intracellular organelles (Rosemblatt and Scales, 1989; Dunn, 1989). Thus, studies of Ca^{2+} fluxes in these vesicles can be used to probe the activity of VDCCs in the absence of intracellular events. In this study, the effect of

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anandamide on the function of VDCCs in rabbit T-tubule membranes has been investigated.

2. Materials and methods

2.1. Preparation of transverse tubule membranes

Microsomal membranes were prepared from the back and hind muscles of small (1–1.5 kg) New Zealand White Rabbits, and T-tubules were isolated by sucrose gradient centrifugation as previously described (Dunn, 1989). The animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care (see Guide to the Care and Use of Experimental Animals, Vol. 1 (2nd ed., 1993) and Vol. 2 (1984)). T-tubule membranes were finally resuspended and equilibrated in low K^+ buffer (10 mM HEPES–Tris, pH:7.4, 145 mM choline chloride, 5 mM potassium gluconate, 0.02% NaN_3), and stored at −86°C. Prior to use, the vesicles were subjected to freeze–thaw cycles to equilibrate intracellular and extracellular ions (Dunn, 1989; Oz et al., 1993).

2.2. Ca^{2+} efflux assay

Membrane vesicles (approximately 0.4 mg/ml) were loaded with $^{45}Ca^{2+}$ by the addition of one-half volume of isotopically diluted $^{45}CaCl_2$ solution in the same buffer to give a final concentration of 5 mM total Ca^{2+} containing approximately 50 μCi/ml $^{45}Ca^{2+}$ (ICN, Irvine, CA, USA). After two freeze–thaw cycles to load Ca^{2+} inside the vesicles, the suspensions were kept on ice until use, which was usually within 1–2 h. A two-step filtration assay (Dunn, 1989) was used to investigate voltage-dependent $^{45}Ca^{2+}$ efflux. Briefly, 25 μl of loaded membranes were first diluted with 975 μl of high K^+ buffer (10 mM HEPES–Tris, pH 7.4, 120 mM potassium gluconate, 30 mM choline chloride, 0.133 mM EGTA) containing 0.1 μM valinomycin and where appropriate, the desired drug. This first dilution is designed to mimic the resting state of the cell by generating an outside negative membrane potential of −80 mV and to reduce the extravesicular (corresponding to intracellular in an inside out vesicle) free Ca^{2+} to less than 100 nM. After 10-min incubation at room temperature, 0.9 ml was removed and applied to a GF/C filter which had been preequilibrated in the same buffer, and dried under vacuum. Excess buffer was removed from the sample under vacuum, and 1 ml of depolarizing buffer (10 mM HEPES–Tris pH 7.4, 5 mM potassium gluconate, 145 mM choline chloride, 0.133 mM EGTA, 0.1 mM valinomycin) was added. Efflux was allowed to continue on the filter for 10 s prior to removal of extravesicular solution under vacuum and rapid washing with two 5 ml volumes of a "stop" solution (10 mM HEPES–Tris pH 7.4, 145 mM choline chloride, 5 mM potassium gluconate, 0.5 mM $LaCl_3$, 30 mM sucrose). Filters with their absorbed membrane vesicles were dried, extracted with 5 ml of Hydroflour™ (National Diagnostics, FL, USA) scintillation fluid and counted for residual entrapped $^{45}Ca^{2+}$.

2.3. Binding studies

Experiments on binding of (+)[^{3}H]PN 200-110 (DuPont-New England) were conducted similar to our earlier studies in these membranes (Dunn et al., 1989). All binding assays were carried out under subdued lighting to minimize ligand photolysis. Briefly, aliquots of membranes (100 μg) were added to different concentrations of radiolabeled ligand to give a final concentration of 0.02 mg/ml T-tubule membranes in a total volume of 0.8 ml. After 60-min incubation at room temperature, 0.4 ml aliquots of each sample were filtered under vacuum through Watman GF/C filters and rapidly washed with 5 ml of ice-cold assay buffer. The filters were dried and extracted in 5 ml of Hydroflour™ (National Diagnostics) scintillation fluid before counting for ^{3}H. Triplicate 50-μl samples of the incubation mixtures were also counted directly for estimations of total binding. Nonspecific binding was estimated from parallel measurements of binding in the presence of 5 μM unlabeled nifedipine. In competition experiments, T-tubule membranes were incubated with 0.5 nM [^{3}H]PN 200-110 at a concentration of 0.02 mg/ml for 1 h with anandamide present in the medium over a concentration range of 0.1–100 μM. Anandamide, nifedipine, Bay K 8644, pertussis toxin, indomethacin and phenylmethylsulfonyl fluoride (PMSF) were from RBI (Natick, CT, USA). SR141716A was purchased from Tocris Cookson (St. Louis, MO, USA). All other chemicals were from Sigma (St.Louis, MO, USA). Drugs were added from stock solutions in Me_2SO and, in order to minimize possible solvent effects, all samples, including controls, contained Me_2SO at the same final concentration (< 0.2%). In flux studies, pertussis toxin was incubated with $^{45}Ca^{2+}$-loaded vesicles for 1 h on ice prior to initiation of repolarization.

2.4. Data analysis

All data are expressed as the arithmetic means and standard errors of the means (S.E.M.) with the number of determinations (n) indicated. In each experiment, at least eight determinations were made of the amount of $^{45}Ca^{2+}$ retained by the vesicles under control conditions, i.e. in the absence of any changes in membrane potential and in the absence of drugs. The mean counts per minute (cpm) was first calculated from control determinations and then normalized to 100%. The cpm of each determination was also normalized to the mean cpm to calculate the SEM for each control. Data obtained under other control conditions are expressed as a percentage of control values. Statistical evaluation of data were made using ANOVA method. For

data analysis and calculation of the IC_{50} values and slope factors, computer fitting software Origin™ (Microcal Software, MA, USA) was used.

3. Results

3.1. Effect of anandamide on voltage dependent Ca^{2+} fluxes

Fig. 1A shows the results of experiments carried out using the two-step protocol described in the method section. Under control conditions (5–5–5 mM K^+ i.e. in the absence of changes in membrane potential) there was no efflux of $^{45}Ca^{2+}$ from the membrane vesicles. Upon exposure of vesicles to depolarizing conditions for 10 s (5–120–5 mM K^+), the amount of $^{45}Ca^{2+}$ remaining in the vesicles was reduced to approximately 30–35% of control values. Both nifedipine (10 μM) and La^{3+} (1 mM) significantly inhibited the flux responses without affecting the content of $^{45}Ca^{2+}$ in the vesicles under control circumstances (C + La and C + Nif in Fig. 1A).

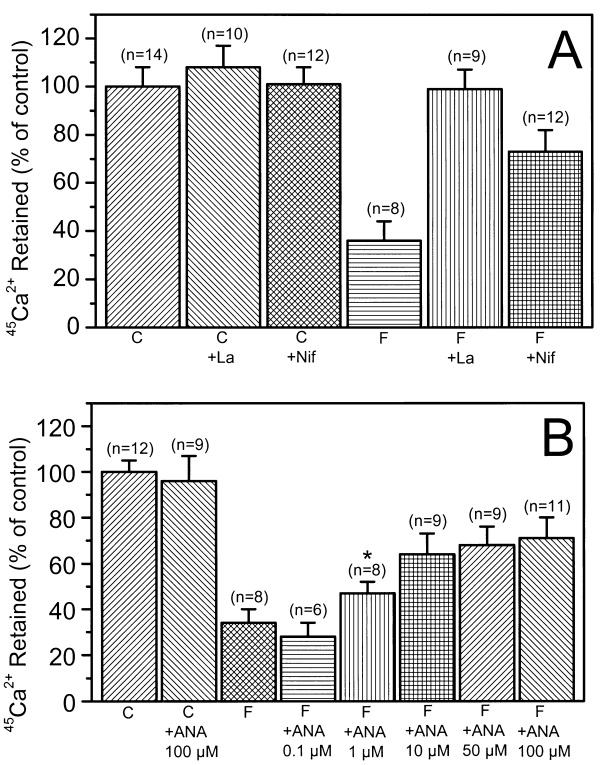

Fig. 1. Induction and pharmacological characterization of $^{45}Ca^{2+}$ flux responses through T-tubule membranes. (A) Effects of membrane potential, inorganic Ca^{2+} channel blocker, La^{3+} (1 mM) and calcium channel blocker nifedipine (10 μM) on $^{45}Ca^{2+}$ effluxes in T-tubule membrane preparations. (B) Effects of increasing concentrations of anandamide on $^{45}Ca^{2+}$ flux responses. The numbers of experiments (n) are presented on top of each column. Vertical lines on top of the columns represents the S.E.M. Statistical significance at the level of $P < 0.05$ was presented with *. C, control conditions; F, efflux conditions; La, lanthanum; Nif, nifedipine; ANA, anandamide.

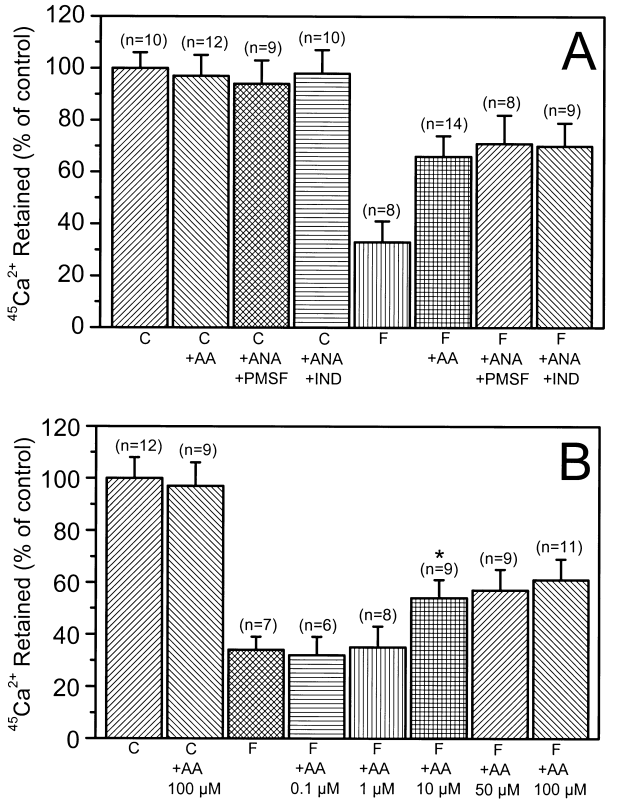

Fig. 2. Effects of drugs related to anandamide metabolism on $^{45}Ca^{2+}$ flux responses through T-tubule membranes. (A) Effects of indomethacin and PMSF on the inhibition of flux responses by anandamide. (B) Effects of increasing concentrations of arachidonic acid on $^{45}Ca^{2+}$ flux responses. The numbers of experiments (n) are presented on top of each column. Vertical lines on top of the columns represents the S.E.M. Level of significance at $P < 0.05$ was presented with *. C, control conditions; F, efflux conditions; ANA, anandamide; AA, arachidonic acid; IND, indomethacin.

Under these experimental conditions, the effect of anandamide was studied over a concentration range of 0.1–100 μM. The effects of 10-min incubation of T-tubule membrane preparations with 0.1, 1, 10, 50 and 100 μM anandamide on $^{45}Ca^{2+}$ efflux responses are illustrated in Fig. 1B. Under control conditions, anandamide, at the highest concentration used (100 μM), had no effect on the amount of $^{45}Ca^{2+}$ retained in T-tubule vesicles. Anandamide at the concentration of 0.1 μM, did not cause a significant change in flux responses. However, in the concentration range of 1–100 μM, anandamide significantly inhibited the amount of $^{45}Ca^{2+}$ retained in vesicles. For example, in the absence and presence of 10 μM anandamide, the amount of $^{45}Ca^{+2}$ retained after depolarization of the vesicles was $34 \pm 6\%$ and $66 \pm 9\%$ of controls, respectively. The difference between these values was statistically highly significant ($P < 0.01$, ANOVA, $n = 8$).

At the concentration of 10 μM, arachidonic acid, a putative metabolite or precursor of anandamide, mimicked the effect of anandamide on flux responses (Fig. 2A and

B). There was no further inhibition of fluxes when arachidonic acid (10 μM) and anandamide (10 μM) were applied simultaneously (data not shown, $n = 6$). The amidohydrolase inhibitor, PMSF (0.2 mM), was added to examine the possible hydrolysis of anandamide to arachidonic acid and the cyclooxygenase inhibitor, indomethacin (5 μM) was used to investigate the possible involvement of endogenous prostaglandin synthesis from anandamide. In the presence of PMSF or indomethacin, inhibitory effect of anandamide on the efflux responses was not significantly changed ($P > 0.05$, ANOVA, $n = 8–9$; Fig. 2A). The effect of 10-min incubation of T-tubule membrane preparations with 1, 10, 50 and 100 μM arachidonic acid on $^{45}Ca^{2+}$ efflux responses are illustrated in Fig. 2B. Under control conditions, arachidonic acid, at the highest concentration used (100 μM), had no effect on the amount of $^{45}Ca^{2+}$ retained in the T-tubule vesicles. Arachidonic acid at the concentration of 1 μM, did not cause any change in flux responses. However, in the concentration range of 10–100 μM, arachidonic acid significantly inhibited the amount of $^{45}Ca^{2+}$ retained in vesicles in a concentration-dependent manner. For example, in the presence of 10 μM arachidonic acid, after high K^+ induced depolarization, content of $^{45}Ca^{+2}$ in the vesicles was 54 ± 7% compared to 34 ± 5% in the control samples. The difference between these values was statistically highly significant ($P < 0.01$, ANOVA, $n = 7–9$).

3.2. Effect of anandamide on the binding of [^{3}H]PN 200-110

The high affinity binding of the dihydropyridine class of Ca^{2+} channel ligands has previously been characterized in T-tubule membrane preparations (Dunn, 1989). Fig. 3A illustrates the inhibition of the specific binding of [^{3}H]PN 200-110 to purified T-tubule vesicles in the presence of 10 μM anandamide. Neither PMSF nor indomethacin alone did effect the binding of [^{3}H]PN 200-110 to T-tubule membranes (data not shown, $n = 4–7$). In addition, the effects of co-application of anandamide (10 μM) with either PMSF (0.2 mM) or indomethacin (5 μM) were not significantly different from anandamide alone. However, incubating the membranes with 10 μM arachidonic acid alone did inhibit the specific binding of [^{3}H]PN 200-110 (Fig. 3A).

Both anandamide and arachidonic acid displaced the specific binding of [^{3}H]PN 200-110 from rabbit T-tubule membranes in a concentration-dependent manner (Fig. 3B). In the concentration range of 30–100 μM, anandamide and arachidonic acid completely inhibited the specific binding of [^{3}H]PN 200-110. The IC_{50} values for anandamide and arachidonic acid were 4.4 ± 0.7 and 13.4 ± 3.5 μM, respectively with corresponding slope factors of 1.35 and 0.95.

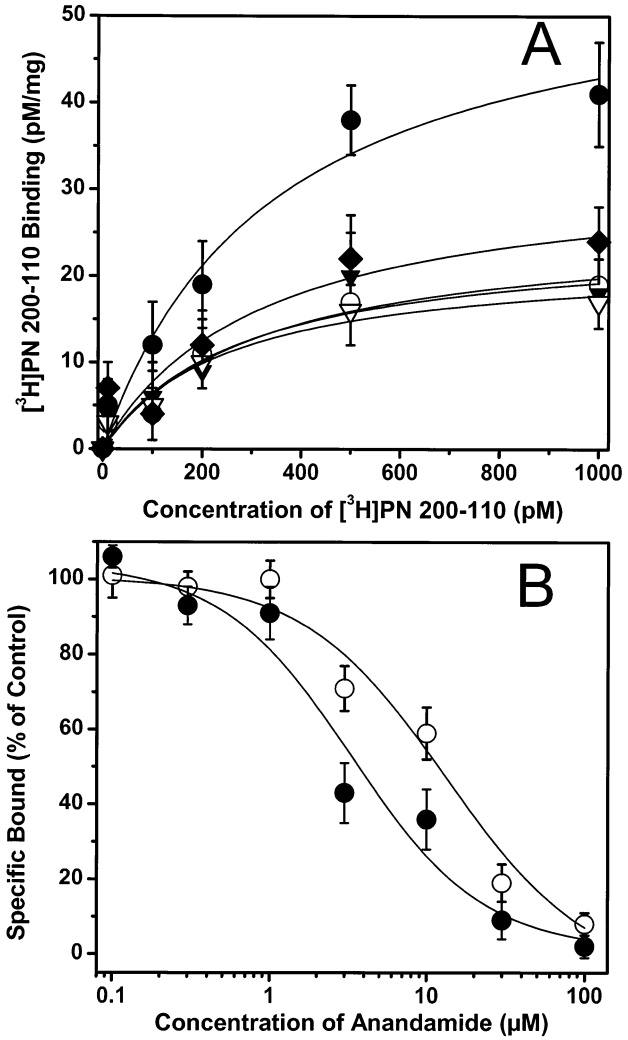

Fig. 3. The specific binding of [^{3}H]PN 200-110 to T-tubule membranes. (A) Specific binding as a function of the concentration of [^{3}H]PN 200-110. Data are presented as the arithmetic means of five experimental measurements in the absence (●) and presence of 10 μM anandamide (○) or 10 μM arachidonic acid (♦). Addition of 0.2 mM PMSF or indomethacin (5 μM) to anandamide in the binding medium is represented by ▽ and ▼, respectively. The incubation time was 60 min at 23°C, pH 7.5. Equivalent samples were incubated with 5 μM of unlabeled nifedipine in order to determine nonspecific binding. (B) The decrease of specific binding of [^{3}H]PN 200-110 to T-tubule membranes by increasing concentrations of anandamide or arachidonic acid. The data are expressed as percentage of control. T-tubule membranes were incubated with 0.5 nM [^{3}H]PN 200-110 at a concentration of 0.02 mg/ml for 1 h with increasing concentrations of anandamide in the medium. Bound and free [^{3}H]PN 200-110 were separated by filtration. Symbols are the means of at least five experiments. Data points for anandamide and arachidonic acid were presented as ● and ○, respectively. Vertical lines represent S.E.M.

3.3. Further studies on the mechanism of anandamide inhibition

Functional interactions between the effects of anandamide and dihydropyridine class antagonist nifedipine or agonist Bay K 8644 on $^{45}Ca^{2+}$ effluxes were also studied.

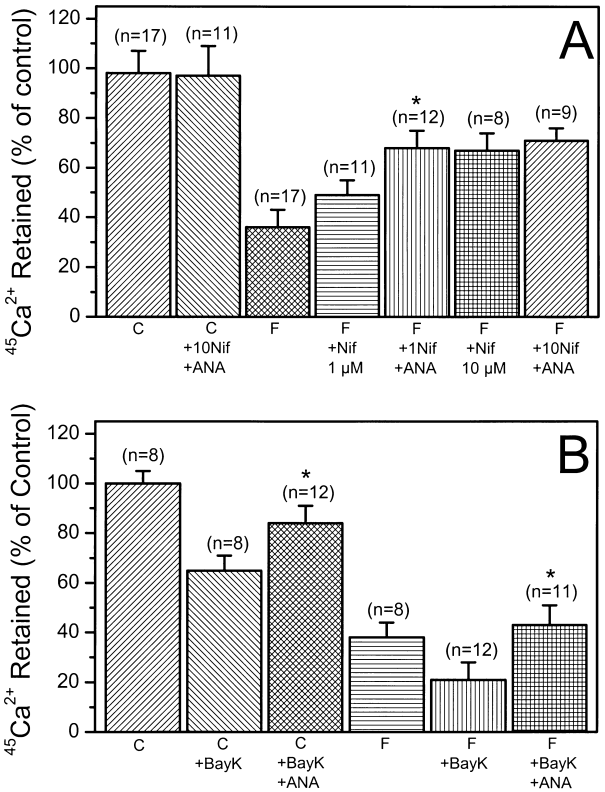

Fig. 4. Functional interaction between the effects of dihydropyridine class VDCC modulators and anandamide on $^{45}Ca^{2+}$ effluxes mediated by depolarizations of T-tubule membrane preparations. (A) In presence and absence of 10 μM anandamide, the effects of incubating T-tubule membranes with 1 and 10 μM of nifedipine on $^{45}Ca^{2+}$ flux responses. (B) Effects of incubating T-tubule membranes with 1 μM of Bay K 8644 in presence and absence of 10 μM anandamide on the amount of retained $^{45}Ca^{2+}$ in control and flux conditions. Vertical lines on top of the columns represents the S.E.M. Statistical significance at the level of $P < 0.05$ was presented with *. Concentrations of 1 and 10 μM of nifedipine were indicated as 1 and 10 in front of the bar labels for nifedipine. C, control conditions; F, efflux conditions; Nif, nifedipine; BayK, Bay K 8644; ANA, anandamide.

In these sets of experiments, in the presence and absence of 10 μM anandamide, T-tubule membrane preparations were incubated with nifedipine (1–10 μM) or Bay K 8644 (1 μM). Fig. 4A represents the results of experiments with 1 and 10 μM nifedipine and the effect of anadamide on nifedipine induced inhibition of flux responses. At the concentrations of 1 and 10 μM, nifedipine alone significantly inhibited the flux responses. The inhibition of flux responses by 1 μM nifedipine, was significantly increased in the presence of 10 μM anandamide (Fig. 4A). In vesicles treated with 1 μM nifedipine, the amount of $^{45}Ca^{2+}$ retained in vesicles were 68 ± 9% and 49 ± 6% of controls, in the presence and absence of anandamide, respectively. There was a statistically significant difference between these values ($P < 0.05$, $n = 11–12$; ANOVA). But, anandamide treatment did not effect the inhibition of effluxes by 10 μM nifedipine. In the presence and absence of anandamide, the amount of $^{45}Ca^{2+}$ retained in vesicles were 71 ± 5% and 67 ± 7% of controls, respectively. There was no statistically significant difference between these values ($P > 0.05$, $n = 8–9$; ANOVA).

Consistent with our earlier results (Dunn, 1989; Oz et al., 1993), Bay K 8644 in the concentration of 1 μM, induced a large efflux of $^{45}Ca^{2+}$ in control conditions (Fig. 4B). The flux responses induced by Bay K 8644 were significantly suppressed in the presence of 10 μM anadamide. In the presence and absence of anadamide, the amount of $^{45}Ca^{2+}$ retained in Bay K 8644 treated control vesicles were 84 ± 7 and 65 ± 6 of controls, respectively. The difference between these values were statistically significant ($P < 0.05$, $n = 8–12$; ANOVA). Further effects of Bay K 8644 on flux responses under depolarizing conditions were significantly decreased by 10-min coincubation of vesicles with anandamide and Bay K 8644 (Fig. 4B). In the presence and absence of anandamide, the amount of $^{45}Ca^{2+}$ retained in Bay K 8644 treated vesicles were 43 ± 7% and 21 ± 6% of controls, respectively. The difference between these values were highly significant ($P < 0.01$, $n = 11–12$; ANOVA).

In order to determine whether anandamide inhibition of VDCCs is mediated by the activation of G-protein coupled cannabinoid receptors, the effect of cannabinoid receptor antagonist, SR141716A and pertussis toxin pretreatment on anandamide inhibition of efflux responses was investigated. Incubating the vesicles with 1 μM SR141716A for 10 min or with 5 μg/ml pertussis toxin for 60 min before the experiments affected neither the controls nor the anandamide-induced inhibition of flux responses (Fig. 5). After the preincubation periods for SR141716A or pertussis toxin, vesicles were tested for the effect of 10-min treatment with 10 μM anandamide on $^{45}Ca^{2+}$ fluxes. In the

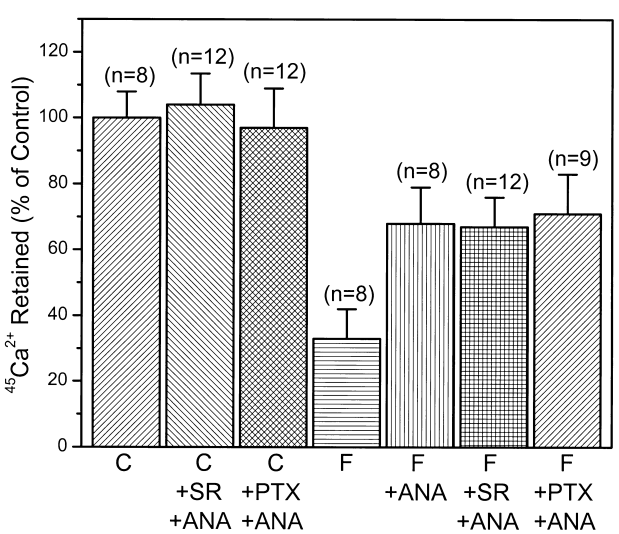

Fig. 5. Effects of SR141716A and pertussis toxin preincubation on the inhibition of flux responses by anandamide. The numbers of experiments (n) are presented on top of each column. Vertical lines on top of the columns represents the S.E.M. C, control conditions; F, efflux conditions; ANA, anandamide; PTX, pertussis toxin; SR, SR141716A.

presence of 10 μM anandamide alone, in the presence of anandamide and SR141716A or anandamide and pertussis toxin, the amounts of $^{45}Ca^{2+}$ retained in the T-tubule vesicles were 68 ± 11%, 67 ± 9% and 71 ± 12% of the controls, respectively. There were no statistically significant differences among these values ($P > 0.05$, $n = 12–14$, ANOVA). In the presence of 1 μM SR141716A alone, there were no significant changes in the amount of $^{45}Ca^{2+}$ retained in vesicles (108 ± 7%) compared to control conditions without SR141716A incubation ($n = 5$; data not shown).

4. Discussion

The main finding of this study is that anandamide, at relatively high concentrations, suppresses the function of VDCCs in T-tubule membranes, and this inhibition is independent of cannabinoid-receptor activation. As is shown in Fig. 1A, these membrane preparations have functional VDCCs i.e., these channels respond to depolarizing voltage changes by opening and are blocked by established inorganic and organic VDCC blockers (Dunn, 1989; Oz et al., 1993). Although the inhibitory effect of anandamide and cannabinoids on neuronal Ca^{2+} channels has been demonstrated in earlier studies (Caulfield et al., 1992; Mackie et al., 1994, 1995; Twitchel et al., 1997), to our knowledge this is the first report indicating that anandamide and arachidonic acid inhibit the function of L-type VDCC in an isolated muscle preparation.

In earlier studies using a variety of cell types, it was found that anandamide, at nanomolar concentrations, causes the release of Ca^{2+} from intracellular stores thus increases the concentration of intracellular Ca^{2+} (for reviews, Di Marzo and Fontano, 1995; Felder and Glass, 1998). Since VDCCs have previously been shown to be modulated by changes in intracellular Ca^{2+} concentrations (Brehm and Eckert, 1978; Feldmeyer et al., 1993; Soldatov et al., 1998; Oz et al., 1998), it remained to be established whether the effect of anandamide on VDCCs is direct or secondary to these changes. Since the T-tubule vesicles used in this study are in an inside-out orientation and are devoid of intracellular organelles (Rosemblatt and Scales, 1989; Dunn, 1989), it is unlikely that the observed inhibitory effect of anandamide on Ca^{2+} fluxes through inside-out oriented vesicles involves changes in intracellular Ca^{2+}. In addition, these membranes are devoid of any plasmalemmal Ca^{2+} pump activity (Dunn, 1989; Rosemblatt and Scales, 1989). In T-tubules, the abundant presence of dihydropyridine binding sites (Fosset et al., 1983; Dunn et al., 1989) and stereospecific modulation of flux responses by dihydropyridines and other class of Ca^{2+} channel antagonists (Oz et al., 1993) have been shown. Using fluorescent voltage sensitive dyes, voltage-changes predicted by the Nernst equation have also been recorded in T-tubule membranes depolarized by changes in extravesicular concentration of K^+ (Dunn, 1989).

Both, anandamide at 1 μM and higher concentrations, and arachidonic acid at 10 μM and higher concentration, significantly inhibited the flux responses in a concentration-dependent manner (Figs. 1B and 2B). Since our attempts to obtain more gradual flux responses, by decreasing depolarization time to 5 s and/or using partial depolarizing conditions were not successful, quantitative aspects of the concentration–response relation could not be studied. It seems that relatively narrow portion of total ion fluxes are carried through L-type VDCCs. This may be due to several factors. Firstly, there is a considerable amount of $^{45}Ca^{2+}$ presumably bond nonspecifically to divalent binding sites on lipid bilayers. The presence of this bound Ca^{2+} is evident with 15–25% of total $^{45}Ca^{2+}$ remained even after depolarization of vesicles pretreated with Bay K 8644 (Fig. 4B). The differential contribution of this bound Ca^{2+} to each batch of the membrane vesicles may be a confounding factor. Secondly, the presence of T-type channels in T-tubules of amphibian and mammalian skeletal muscle have been reported. (Arreola et al., 1987; Garcia and Stefani, 1987; Dirksen and Beam, 1995). Although under prolonged depolarizing conditions employed in this study, T-type channels are likely to be largely inactivated, considerable flux of $^{45}Ca^{2+}$ through these channels in early phase of depolarization may interfere with our results. In fact, nifedipine (10 μM), that completely abolishes the Ca^{2+} currents mediated by L-type VDCCs, without affecting T-type VDCCs in mammalian skeletal muscle fibers (Lamb and Walsh, 1987; Dirksen and Beam, 1995; Patterson et al., 1995) did not block the flux responses completely (Figs. 1A and 4A). The remaining 20–30% of total flux response resistant to inhibition by anandamide or arachidonic acid may reflect the contribution of T-type channels to total fluxes. In line with this conclusion, almost 100% inhibition of flux responses by La^{3+} (Fig. 1A), which effectively blocks both L and T-type VDCCs (Narahashi et al., 1987; Mlinar and Enyeart, 1993; Dirksen and Beam, 1995) may also imply the contribution of T-type VDCCs.

Anandamide has been shown to be partially hydrolyzed to arachidonic acid by a membrane-associated amidohydrolase under the conditions membrane vesicles were incubated (Di Marzo and Fontana, 1995; Jarrahian and Hillard, 1997). In the presence of 0.2 mM PMSF, amidohydrolase inhibitor, or 5 μM indomethacin, cyclooxygenase inhibitor, anandamide-induced inhibition of flux responses were not significantly altered (Fig. 2A), indicating that the metabolites of anandamide hydrolysis were not significantly contributed to effect of anandamide. However, at 10 μM and higher concentrations, arachidonic acid per se did cause a significant inhibition of flux responses (Fig. 2). Since the effect of arachidonic acid on the functional properties of the VDCCs of skeletal muscle, to our knowledge, has not been reported in earlier studies, we can not compare the present results with the findings in literature. But, in both smooth and cardiac muscle preparations,

inhibitory effects of arachidonic acid (1–50 μM) on Ca^{2+} currents mediated through L-type VDCC have been reported (Nagano et al., 1995; Petit-Jacques and Hartzell, 1996; Xiao et al., 1997; see also c.f. Huang et al., 1992).

Experimental evidence suggest that both anandamide and arachidonic acid induced suppression of flux responses are due to the inhibition of L-type VDCCs located in T-tubule vesicles. Firstly, in the presence of anandamide, the inhibitory effect of 1 μM nifedipine on flux responses was significantly increased. In addition, the effect of anandamide on nifedipine induced inhibition was occluded at saturating concentrations of nifedipine (10 μM). These findings may suggest that anandamide and nifedipine are mediated through a common ionic pathway. Secondly, in the presence of anandamide, reversal of the effect of Bay K 8644 on flux responses also suggest that the effect of anandamide is mediated by the inhibition of L-type VDCCs. Thirdly, the La^{3+} sensitive part of the $^{45}Ca^{2+}$ retained in vesicles remained resistant to both nifedipine and anandamide. These findings may suggest that both nifedipine and anandamide specifically effect fluxes mediated by L-type VDCCs and they act through common ionic pathway, presumably by the suppression of fluxes through L-type VDCCs.

The results indicate that the inhibitory effect of anandamide is not sensitive to pertussis toxin treatment. Furthermore, the cannabinoid receptor antagonist, SR141716A did not modulate the effect of anandamide. In recent studies, similar concentrations of anandamide were shown to inhibit K^+ channels, nicotinic acetylcholine receptors and gap junctions and these effects were also found to be insensitive to both SR141716A and pertussis toxin pretreatment (Venance et al., 1995; Oz et al., 1996; Poling et al., 1996). In some of the earlier studies, the effects of anandamide on ion channels were found to be mediated by the activation of cannabinoid receptors while, in others, direct effects of anandamide on ion channels were demonstrated (for a recent review; Felder and Glass, 1998). In neuronal cell lines, cannabinoid receptor mediated and pertussis toxin sensitive inhibition of Ca^{2+} channels in nanomolar concentration range of anandamide have been demonstrated (Mackie et al., 1994, 1995; Twitchel et al., 1997). Our study indicates that in addition to its receptor mediated effects at lower concentrations, direct inhibition of L-type VDCCs can also be observed at equal to or higher than 1 μM concentrations of anandamide.

The ability of anandamide to inhibit the specific binding of the radioligand [^{3}H]PN 200-110 to T-tubule membranes suggests that anandamide may interact with dihydropyridine binding sites in the VDCC. This inhibition was not affected by the presence of PMSF in the medium, indicating that the effects are not due to arachidonic acid arising from the hydrolysis of anandamide. Similarly in cortical, cardiac (Johnson et al., 1993) and forebrain (Jarrahian and Hillard, 1997) membranes, no effects of PMSF or hydrolyzed products of anandamide were found. However, arachidonic acid itself produced effects that are similar and comparable to the effects of anandamide. Interaction of both anandamide and arachidonic acid on dihydropyridine binding sites have been observed in studies of muscle and brain membranes (Johnson et al., 1993; Shimsue et al., 1996; Jarrahian and Hillard, 1997). Anandamide may act through several mechanisms. The presence of a low-affinity binding site for dihydropyridines on VDCCs has been demonstrated in T-tubule membranes (Dunn and Bladen, 1991, 1992). Allosteric interaction of anandamide with this low-affinity binding site may mediate it's functional effects on VDCCs. Anandamide may also act through a binding site located on phospholipids. In earlier studies, it was shown that the binding of dihydropyridines and the function of VDCCs were affected by phospholipids and lipids added to assay medium (Glossmann and Ferry, 1983; Coronado, 1987).

Recently, it was demonstrated that anandamide, at similar concentration ranges, can act directly on gap junctions of astrocytes (Venance, 1995), on ligand-gated ion channels expressed in oocytes (Oz et al., 1996) and on various types of K^+ channels expressed in mammalian cell lines (Poling et al., 1996). In conclusion, present results demonstrating a suppression of voltage-dependent Ca^{2+} fluxes in skeletal muscle T-tubules suggest that at the concentrations of 1 μM and higher, cannabinoid receptor independent effects of anandamide should also be considered.

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