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EFFECTS OF CALCIUM CHANNEL ANTAGONISTS ON THE RELEASE OF PROSTAGLANDIN E_2 FROM METABOLICALLY STRESSED MUSCLE

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Abstract—Calcium influx plays a critical role in the activation of the arachidonic cascade in muscle damage. We examined the effects of L-type calcium channel antagonists on the release of prostaglandin E_2 (PGE_2), a bioactive metabolite of arachidonic acid metabolism, from skeletal muscle. The basal release of PGE_2 was not affected by calcium channel inhibitors, such as nifedipine and verapamil. The release of PGE_2 induced by dinitrophenol, an uncoupler of oxidative phosphorylation, was abolished by nifedipine and verapamil at 50 and 150 μM, respectively. It was not necessary to include the calcium channel blockers in the medium before or at the time of dinitrophenol stimulation to produce the effect on PGE_2 release. The release of PGE_2 was prevented for as long as calcium channel blockers were present in the medium after the dinitrophenol stress.

Key words: skeletal muscle; 2,4-dinitrophenol; prostaglandin E_2; calcium channel blockers; nifedipine; verapamil

AA† and its cyclooxygenase product PGE_2 are released in response to cell damage due to various causes such as ischemia, hypoxia, and metabolic stress in many tissues including brain, heart and skeletal muscle [1–5]. We previously demonstrated that there are two components to PGE_2 release from muscle [5, 6]. Basal release is not affected by calcium, whereas PGE_2 release induced by metabolic stress is calcium dependent.

Various calcium channels play pivotal roles in the elevation of intracellular calcium in several pathophysiological events [7]. Calcium channel antagonists, especially voltage-sensitive L-type calcium channel blockers, have been used clinically, but little is known about the mechanisms of drugs and disease-induced calcium channel regulation [7–9]. Recent studies demonstrated the presence of both high and low affinity binding sites for L-type calcium channel antagonists in skeletal muscle [10]. However, physiological functions of calcium channels in muscle remain obscure.

In the present report, rat epitrochlearis muscle was metabolically stressed with DNP to induce PGE_2 release. We examined the effects of two L-type calcium channel antagonists, nifedipine (1,4-dihydropyridine) and verapamil (phenylalkylamine), on the release of PGE_2 from muscle.

MATERIALS AND METHODS

Materials. [^{3}H]PGE_2 (154 Ci/mmol) was purchased from New England Nuclear. PGE_2, anti-PGE_2 antiserum, bovine serum albumin, TMPD and DNP were purchased from ICN Biomedicals. Nifedipine and verapamil were provided by Dr. Susan Dunn of the University of Alberta.

Muscle preparation. Male Sprague–Dawley rats (150–200 g) were killed by cervical dislocation. Epitrochlearis muscle [5, 11], which weighs about 30 mg, was rapidly dissected, mounted in special holders between two stainless steel clips, and suspended in 3 mL of oxygenated (95% O_2 and 5% CO_2) Krebs–Ringer buffer, pH 7.4, at 37°. The muscle was allowed to equilibrate with the medium over a period of 15 min. A twitch was produced by electrical stimulation using a Grass stimulator. The tension was recorded using a strain gauge connected to a strip chart recorder. Muscles that produced less than 6 g tension were rejected, as was any control muscle that fell below 80% of the initial force over the subsequent 15 min. The medium was changed at the start of the experiment and every 30 min thereafter, and various agents were added as indicated below. The twitch tension in response to a single maximal stimulation was measured every 30 min just before the medium was changed. Samples were collected every 30 min and stored at −70° for PGE_2 assay. At the end of the experiments, the muscles were frozen.

Addition of various agents. During the treatment period, the muscles were incubated in the presence of DNP (40 μM) for 30 min (between 30 and 60 min, see Fig. 1). In some experiments nifedipine (5 or 50 μM) or verapamil (50 or 150 μM) was present: before, during and 30, 60 and 90 min after DNP treatment. Nifedipine was dissolved in ethanol prior to addition to the medium, and the same amount of ethanol (less 0.2% final concentration) was added to control groups. For control studies, the experiment

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† Abbreviations: AA, arachidonic acid; PGE_2, prostaglandin E_2; DNP, 2,4-dinitrophenol; TMPD, tetramethylphenylenediamine; and PGH synthase, prostaglandin H synthase.

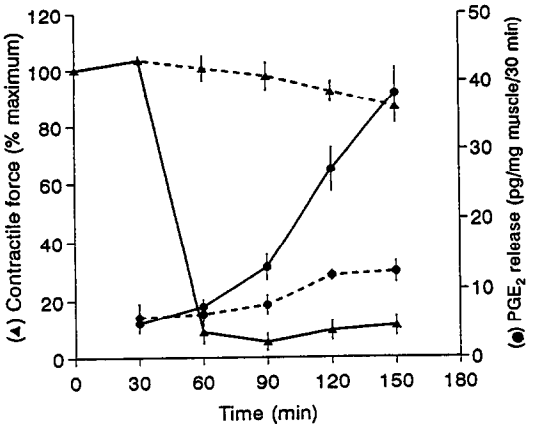

Fig. 1. Muscle contractile force (▲) and PGE_2 release (●) measured at different time intervals. Muscles were treated in the absence (- - - - -) and presence of DNP (——). DNP (40 μM) was present in the medium between 30 and 60 min. Values are means ± SEM, N = 6.

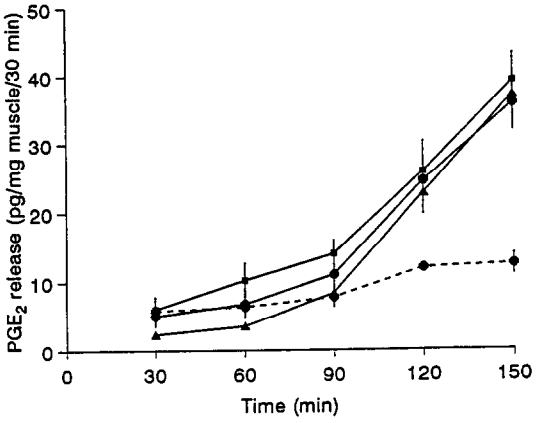

Fig. 2. Efflux of PGE_2 from control muscles (- -●- -) and DNP-treated muscles (—●—). DNP (40 μM) was present in the medium between 30 and 60 min. Values are means ± SEM, N = 6. DNP-induced PGE_2 release was compared with DNP-induced PGE_2 release in the presence of 50 μM nifedipine, 0–30 min (■) and 30–60 min (▲).

was carried out in the absence of DNP or calcium channel blockers.

PGE_2 assay. PGE_2 was measured by radioimmunoassay as described earlier [5]. Neither nifedipine (50 μM) nor verapamil (150 μM) interferes with radioimmunoassay. The concentration of PGE_2 was expressed as picograms of PGE_2 per milligram of muscle wet weight per 30 minutes.

PGH synthase/peroxidase assay. Fresh muscle was homogenized at 4° in 2 mL of 50 mM Tris–HCl buffer, pH 8.0, containing 2 mM EDTA, 2 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 40,000 *g* for 30 min, and the supernatant was collected. The peroxidase activity of PGH synthase was measured by a modification of a standard spectrophotometric method [12]. Briefly, the assay mixture contained 5 μL of the muscle supernatant, 0.35 mM TMPD, 100 mM Tris–HCl buffer, pH 8.0, to a final volume of 1 mL. In some experiments, nifedipine (50 μM) or verapamil (150 μM) was included in the assay mixture. The reaction was initiated by the addition of 0.9 μmol H_2O_2, and the change in the absorbance was recorded at 609 nm for 30 sec to monitor the oxidation of TMPD. The above studies were performed using aliquots of microsomal fraction of the same epitrochlearis muscle. One unit of enzyme activity is defined as 1 μmol TMPD oxidized/min using an extinction coefficient of 13.5/mM TMPD oxidized [13].

Statistical analysis. Unless otherwise indicated, six muscles were included in each treatment group. The data are presented as the mean ± SEM. Statistical significance was determined using the Mann–Whitney nonparametric two sample test (Number Cruncher Statistical System software program, Kaysville, UT, 1990).

RESULTS

Twitch tension. In control muscles, twitch tension was always maintained at greater than 80% of the initial tension at the end of the experiments (Fig. 1). In contrast, DNP-treated muscles showed a rapid decline in twitch tension (5.0 ± 2.0% of the initial value). In the absence of DNP, muscles treated with calcium channel antagonists, such as nifedipine or verapamil, showed a steady decline in the twitch tension (data not shown).

PGE_2 release. The efflux of PGE_2 from muscle was maximal at the end of the experiment and was elevated markedly in the case of DNP treatment (Fig. 1). Treatment of the muscles throughout the experiment with nifedipine prevented the DNP-induced release of PGE_2. At 150 min, this release was 21.3 ± 1.7 and 14.0 ± 1.2 pg/mg muscle/30 min, in the presence of 5 and 50 μM nifedipine, respectively. Nifedipine alone had no effect on control PGE_2 release (data not shown). Since 50 μM nifedipine abolished the DNP-stimulated PGE_2 release, we further examined the effect of this calcium channel blocker by addition of this compound at different times before, during and after DNP treatment; the results are summarized in Figs. 2 and 3. DNP-stimulated release of PGE_2 was not blocked when the muscles were treated with nifedipine before and during DNP treatment (Fig. 2). The presence of nifedipine after DNP stimulation prevented this release only as long as it was present in the medium (Fig. 3). Thus, the removal of nifedipine from the incubation 30 or 60 min after the end of DNP treatment resulted in a significant rise in PGE_2 release at 150 min.

To determine whether another type of L-type calcium channel blockers is as effective as nifedipine (a dihydropyridine), we tested the effect of verapamil (a phenylalkylamine) on PGE_2 release. Treatment of muscles throughout the experiment with verapamil (150 μM) inhibited DNP-induced PGE_2 release (21.8 ± 3.4 pg/mg muscle/30 min), although, in contrast to nifedipine, 50 μM verapamil could not prevent this release (41.6 ± 2.9 pg/mg muscle/30 min). Figure 4 shows that the continuous presence of verapamil (150 μM) following DNP treatment significantly inhibited PGE_2 release at 150 min. Like nifedipine, the withdrawal of verapamil from the

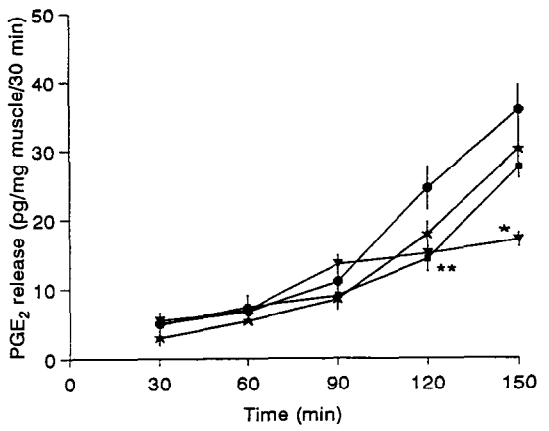

Fig. 3. Time-dependent inhibition of the DNP-induced PGE_2 release by nifedipine (50 μM). DNP (40 μM) was present in the medium between 30 and 60 min in each experiment. DNP-induced PGE_2 release (—●—) was compared with DNP-induced PGE_2 release in the presence of nifedipine, 60–90 min (★), 60–120 min (■), and 60–150 min (▼). Values are means ± SEM (at least six muscles in each group). Key: (*) $P < 0.003$ between nifedipine treatment 60–120 min and 60–150 min, and (**) $P < 0.03$ between DNP treatment and DNP plus nifedipine treatment either 60–120 min or 60–150 min.

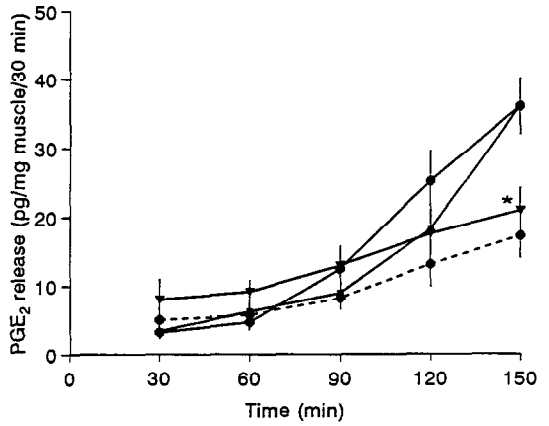

Fig. 4. Effect of verapamil on the release of PGE_2 from control muscles (- -●- -) and DNP-treated muscles (—●—). DNP (40 μM) was present in the medium between 30 and 60 min. DNP-induced PGE_2 release was compared with DNP-induced PGE_2 release in the presence of 150 μM verapamil, 60–120 min (■) and 60–150 min (▼). Values are means ± SEM, N = 6. Key: (*) $P < 0.03$ between verapamil treatment 60–120 min and 60–150 min.

incubation 60 min after DNP treatment caused a significant rise in the PGE_2 release over the next 30 min.

PGH synthase/peroxidase activity. In the absence and presence of nifedipine (50 μM) or the presence of verapamil (150 μM), the PGH synthase/peroxidase activity of microsomal fraction of rat epitrochlearis was 7.8, 6.7, and 7.9 nmol/min, respectively.

DISCUSSION

Voltage-dependent calcium channels regulate calcium influx and thereby contribute to calcium signalling in many cells [7, 14]. Among the four different types (L, P, N, and T), the L-type calcium channel has been studied extensively because of its detection in membrane preparations from a variety of excitable tissues including smooth muscle, cardiac muscle, skeletal muscle and brain [9]. Three classes of L-type calcium channel antagonists such as 1,4-dihydropyridines, phenylalkylamines and benzothiazepines, respectively, exemplified by nifedipine, verapamil and diltiazem, have been widely used clinically in the treatment of diseases [7, 8]. In spite of much effort, the biochemical and molecular basis of action of these calcium channel drugs is not well understood. Nothing is known about the interaction of calcium channel blockers with calcium-triggered events such as activation of the AA cascade. The production of PGE_2, an oxidized metabolite of AA, is one of the earliest biochemical responses to ischemia, hypoxia and metabolic stress [1, 3, 5,15], and PGE_2 is a mediator of several biochemical and pathophysiological events [16–18]. In the present study, an *in vitro* preparation of the rat epitrochlearis muscle was used, as described previously [11]. This small muscle can be dissected from insertion to insertion and is thin enough that satisfactory oxygenation is possible by diffusion. Muscle function was evaluated by the twitch tension evoked by a single supramaximal electrical stimulus. We have used this muscle model and have shown that PGE_2 release stimulated by DNP is dependent on calcium [5, 6]. Since the production of PGE_2 is regulated by the availability of free AA release by the action of phospholipases [19], and these are activated, in turn, by the influx of extracellular calcium [20], we examined the effects of two different L-type calcium channel blockers, namely nifedipine and verapamil, on the release of PGE_2.

The use of DNP stimulation in the rat epitrochlearis muscle model resulted in an immediate fall in twitch tension followed 30–60 min later by an increased release of PGE_2 (Fig. 1). The delay between the fall of muscle contractile function and a rise in PGE_2 release suggests that there may be a window of time (30–60 min in our model) when muscle fibres are injured, but salvageable, a time when therapeutic interventions may be of benefit [6]. Previous studies by Jackson *et al.* [3] demonstrated that the resultant increase in intracellular calcium by DNP treatment increased the production of PGE_2 via phospholipase A_2 and cyclooxygenase pathways in muscle. The present study revealed that it is not necessary to include the calcium channel antagonists in the medium before or during the time of DNP stimulation to produce the effect on PGE_2 release (Fig. 2). It is noteworthy that this DNP-stimulated PGE_2 release was reduced markedly by both calcium channel blockers for as long as these compounds were present in the incubation after the DNP stimulation up to 90 min, the limit of our experiment (Figs. 3 and 4). In light of previous findings [3, 5], the present study suggests that both nifedipine and verapamil block the entry of calcium from extracellular medium by interacting with muscle calcium channel proteins [9, 10] and inhibit a process that elevates PGE_2 production.

In skeletal muscle, cardiac muscle and neuronal

tissue, the concentration of calcium antagonists required to affect calcium channel and function are several magnitudes higher (micromolar range) than those required to saturate high affinity binding sites in isolated membrane preparation (nanomolar range) [10, 21]. Calcium channels carry multiple binding sites and interact differentially with different classes of antagonists [9, 10, 21, 22]. Thus, different affinities and occupancy of these sites lead to different responses. In a recent study, as high as 300 μM verapamil was used to effectively inhibit the calcium-activated prostaglandin $F_{2\alpha}$ production from hippocampal mossy fiber synaptosomes [23]. It is not known whether nifedipine or verapamil inhibits DNP-induced PGE_2 release by interacting with additional components [24] apart from blocking their specific receptors. In this regard, our study indicated that PGH synthase, a key rate-limiting enzyme in the synthesis of PGE_2, was not inhibited by verapamil (150 μM) and inhibited less than 14% by nifedipine (50 μM). Despite extensive interaction, the physiological roles of the L-type calcium channel protein(s) in skeletal muscle remain to be established, and the mechanism by which these channel-blocking drugs interact with the complex network of the AA cascade is currently unresolved. Further experiments will be needed to delineate this.

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