Contraction of isolated smooth muscle cells by ionophore A23187

(calcium/carbamylcholine chloride/X537A/calcium pools)

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ABSTRACT Ionophore A23187 causes contraction of smooth muscle cells isolated from the stomach muscularis of *Bufo marinus*. The peak response is reached within 10 sec of ionophore addition and then decays to a lesser, sustained contraction. The initial phase of contraction is maximal at A23187 concentrations greater than 5×10^{-6} M and half maximal at 5×10^{-7} M. Chelation of extracellular calcium by ethyleneglycol-*bis*(β -aminoethylether)-*N,N'*-tetraacetic acid (EGTA) does not affect the initial contraction produced by A23187, whereas it inhibits completely the sustained, secondary phase. Addition of calcium in excess of EGTA restores this phase rapidly and fully to control levels of contraction. A23187 produces a maximal, rapid contraction of muscle cells previously depolarized by incubation in high potassium medium. The ionophore also contracts isolated strips of stomach smooth muscle. A23187 appears to produce contraction of the isolated smooth muscle cells by transmembrane transport of calcium into the cytoplasm from both intracellular and extracellular sources.

It is well established that contraction of skeletal muscle is initiated by calcium released from the sarcoplasmic reticulum. The relative importance of intracellular versus extracellular calcium stores in providing calcium for contraction of smooth muscle, however, is a matter of current debate (1-5). Since antibiotic A23187 transports calcium across a number of membranes (6-12), and shows an apparent selectivity for divalent over monovalent cations (6, 7, 13), it appeared to provide a relatively specific and direct means to facilitate calcium release from stores available for smooth muscle contraction.

A23187 was reported to produce, however, only a weak, highly calcium-dependent contraction of rabbit aortic ring muscle (14). Since studies of the effect of A23187 on intact muscle tissue may be complicated by permeability barriers and effects of the ionophore on other tissue components (nerves, blood vessels), we examined the effects of A23187 on isolated gastric smooth muscle cells (15, 16). The present results show that A23187 produces a rapid, large contraction which can be supported by either intra- or extracellular calcium. A preliminary account of these results has been presented (17).

MATERIALS AND METHODS

Smooth muscle cells were isolated from slices of stomach muscularis of *Bufo marinus* incubated with trypsin and collagenase (15). Only preparations in which the cells appeared relaxed and in which carbamylcholine chloride (carbachol) induced a vigorous contraction when viewed under a phase contrast microscope were used. The cells were incubated at 22° in amphibian Ringer's solution containing 1.8 mM CaCl_2 and 1.0 mM MgCl_2 (15). In some experiments cells were depolarized by a 15-min incubation in a medium in

which potassium was increased from 3.0 to 75.2 mM, and to maintain isotonicity sodium was decreased from 96.3 to 24.1 mM. A23187, X537A (14, 18), or carbachol were added to 10-ml aliquots of a cell suspension (approximately 5×10^5 cells), and after a specific time, the cells were fixed by addition of acrolein to a final concentration of 1%. Small particulate debris and acrolein were removed by sedimenting the fixed cells for 10 min, at $100 \times g$. The pellet was resuspended in 0.8% NaCl, washed four times as indicated above, and suspended finally in 10 ml of this medium.

The contractile state of these cells was assessed with a Coulter Counter (19). Contraction is associated with increased cell diameter (15) which is measured as an increase in the height of the pulse produced by a particle (19). Pulse height histograms were generated from 10^4 cells in each suspension. The increased percentage of larger amplitude pulses produced by a treated group of cells relative to an untreated control is then a measure of the degree of contraction of cells exposed to different agents. For each cell suspension, the maximal contractile response was defined as 100% and all other responses were expressed relative to this.

Additions to incubation flasks were made from the following stock solutions: carbachol, 55 mM in amphibian Ringer's solution; A23187, 9.6 mM in anhydrous methanol; X537A, 8.2 mM in methanol; and ethyleneglycol-*bis*(β -aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), 0.1 M in glass distilled water, brought to pH 7.4 with triethanolamine. Ionophore solutions were sonicated in an ultrasound bath prior to use to optimize solution of the antibiotics. Methanol was chosen as the solvent since it had no apparent effects on the contractile state of cells up to a concentration of 0.5%, vol/vol, whereas other solvents tested (dimethylsulfoxide, ethanol, dimethylformamide, isobutanol) either contracted or relaxed the cells. Reagent grade acrolein and EGTA were obtained from Eastman Organic Chemicals, Rochester, N.Y., and carbamylcholine chloride was obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Comparison of effects of A23187 and carbachol on isolated smooth muscle cells

A23187 initiated a contraction of isolated smooth muscle cells in every preparation examined when the ionophore concentration was 5×10^{-7} M or greater. The photomicrographs in Fig. 1 show representative cells from three aliquots of a single suspension which were untreated or exposed to concentrations of A23187 or carbachol that routinely yielded a maximal contraction. The drug-treated cells are shorter and have larger diameters in comparison to the long, fusiform shape of control cells. The contractile state of cells in these same three aliquots was quantitated with the Coulter Counter and the results are presented in Fig. 2. The

Abbreviations: EGTA, ethyleneglycol-*bis*(β -aminoethyl ether)-*N,N'*-tetraacetic acid; carbachol, carbamylcholine chloride.

FIG. 1. Photomicrographs of representative groups of treated and untreated cells from a suspension of isolated smooth muscle cells. Prior to fixation, the cells on the left were treated with carbachol (1×10^{-5} M) for 15 sec, and those on the right were treated with A23187 (5×10^{-6} M) for 10 sec. The cells in the center were untreated before fixation. Phase contrast, $\times 72$.

shifts of the pulse height histograms generated by cells exposed to A23187 or carbachol reflect the greater percentage of cells with increased diameters due to contraction. In this experiment, 25.3% of the pulses produced by A23187-treated cells were larger than those produced by control cells; similarly, 32.3% of the pulses generated by cells exposed to carbachol were of larger amplitude than control. In a series of 18 experiments, the ratio of maximal responses, A23187/carbachol, was 1.06 ± 0.08 (SEM).

Sensitivity and kinetics of contractile response produced by A23187

Contraction of smooth muscle cells by various concentrations of A23187 routinely reached a maximum within 10 sec of ionophore addition. The dose response curve of this effect is shown in Fig. 3. Half maximal contraction occurred at approximately 5×10^{-7} M A23187, and the maximal response was observed at 5×10^{-6} M (or greater) ionophore. Although the peak response at 10 sec was highly dependent on the concentration of A23187, a sustained, secondary contraction of lesser, but relatively constant magnitude occurred at ionophore concentrations between 5×10^{-7} and 1×10^{-5} M (Fig. 4). This secondary phase was reached within one to 3 min of A23187 addition and remained essentially unaltered for the additional time investigated (Fig. 4).

Effects of EGTA on A23187-induced contraction

One of several possible explanations for the biphasic nature of the contractile response of smooth muscle cells to A23187 was the interaction of the ionophore with more than one calcium pool. To investigate the possible contribution of extracellular calcium in the response to A23187, we preincubated cells, for various periods of time, with a concentration of EGTA sufficient to chelate all the extracellular calcium (3 mM EGTA). The rapid, initial contraction produced by A23187 was not significantly affected by exposure of cells to EGTA for up to 10 min before ionophore addition, whereas the peak response to carbachol (15 sec) was inhibited 54% by just a 10-sec preincubation with chelator (Fig. 5). A one hour preincubation of cells with EGTA prevented completely the rapid contractions produced by both A23187 and carbachol. These results suggested that the initial response of cells to A23187 could be produced solely by the action of the ionophore on an internal calcium pool, whereas the carbachol-induced contraction was at least partially dependent on extracellular calcium.

The sustained phase of the A23187-induced contraction (3-min time point) was inhibited completely when EGTA was added 10 sec prior to ionophore (Fig. 5). Thus, this phase was supported by calcium from either or both the ex-

FIG. 2. Pulse height histograms generated from isolated smooth muscle cells after exposure to A23187 or carbachol. Histograms are of the cell suspensions shown in Fig. 1.

FIG. 3. Dose response curve of isolated smooth muscle cell contraction by A23187. Cells were exposed to ionophore for 10 sec before fixation. The maximal shift in the pulse height histogram in each experiment was designated as 100% and the response to other concentrations of ionophore expressed relative to it. Points are the mean $\pm$ the standard error of the mean (SEM) of the number of different cell suspensions indicated in parentheses. Zero to maximal response to A23187 occurred over a concentration range of less than two orders of magnitude.

tracellular medium and internal stores which were depleted effectively by EGTA *plus* ionophore during the 3-min incubation shown in Fig. 5. When extracellular calcium was restored to control levels at this point, the contractile response returned within 10 sec to the level usually seen 3 min after the addition of A23187 (Fig. 6). This observation shows that a contractile state similar in magnitude to that observed 3 min after A23187 addition can be supported by extracellular calcium, since it is unlikely that an internal calcium store would be replenished within 10 sec under these conditions.

Further characterization of A23187-induced contraction

To determine if the contraction produced by A23187 required a normal membrane potential, we incubated cells for 15 min in a high potassium medium. This treatment leads to a sustained depolarization of the cell membrane (20). The initial contraction induced by high external potassium is similar in magnitude to that produced by A23187, but decays to 35% of its peak value after 15 min. Addition of A23187 at this point produced a contraction of the usual time course and magnitude, suggesting that normal membrane polarization is not necessary for response of the muscle cells to the ionophore.

A23187 produced a rapid and large contraction of isolated strips of stomach muscularis from *Bufo marinus*, in contrast to a reported weak effect on strips of rabbit aortic ring muscle (14). On the other hand, X537A, at a concentration of 5×10^{-6} M, produced a strong contraction of aortic ring smooth muscle (14), but induced only a weak contraction of the isolated stomach smooth muscle cells (25% of the response to an equal concentration of A23187).

DISCUSSION

Contraction of isolated smooth muscle cells by A23187 results almost certainly from the ionophore's ability to transport calcium across membranes in response to a concentra-

FIG. 4. Time course of the contractile response induced by various concentrations of A23187. *Abcissa*: minutes after ionophore addition. *Ordinate*: percent of observed maximal response to the optimally effective concentration of A23187 in each cell suspension. Points in B and D are the average of two experiments; in A and C, they are the mean $\pm$ SEM for the number of experiments in parentheses. The concentration of A23187 is indicated in the upper right corner of each panel.

tion gradient of this cation (6–12). If it is assumed that the time course of the contractile response reflects the concentration of calcium in the cytoplasm, then addition of A23187 is followed by a rapid rise in cytoplasmic calcium, which then decays to a lower steady-state level.

The rapid, large initial contraction of smooth muscle cells by A23187 can be produced solely by ionophore-induced release of calcium from internal, EGTA-inaccessible stores. Thus, A23187 appears to enter intracellular membranes very rapidly. Our results suggest that A23187 also resides in the plasma membrane 10 sec after ionophore addition, and so transfer of extracellular calcium down its concentration gradient into the cell would also contribute to the initial contraction. Localization of A23187 in the plasma membrane at this early time is supported by studies with cells exposed to EGTA for 60 min. Although these cells fail to contract when A23187 is added (presumably due to depletion of internal calcium), the 10-sec contractile response is restored partially ($56 \pm 9\%$) if extracellular calcium is returned to control levels immediately prior to ionophore addition. In these experiments, it seems likely that A23187 raises cytoplasmic calcium concentrations by transferring extracellular calcium across the plasma membrane.

The sustained secondary phase of the contraction produced by A23187 may depend largely on external calcium, since its complete inhibition by EGTA can be reversed rapidly (within 10 sec) following restoration of extracellular calcium to control levels. This brief period of time is probably insufficient to replenish an internal calcium store, and the reversal by extracellular calcium suggests again that A23187 resides in the plasma membrane. According to our assumption, relaxation of the initial contraction would result from a decrease in cytoplasmic calcium concentration. It may be suggested that a lower cytoplasmic calcium could be produced by either or both of the following mechanisms: (1) depletion of internal calcium stores due to ionophore-mediated efflux, not matched by reuptake processes; and (2) activation of outwardly directed calcium "pumping mechanisms" at the plasma membrane. Relaxation does not result from the contractile mechanism becoming refractory to sustained intracellular calcium levels, since carbachol, added during

FIG. 5. Effect of EGTA on A23187- and carbachol-induced contractions. *Abcissa*: minutes of preincubation of cells with 3 mM EGTA before exposure to A23187 for 10 sec or 3 min, or carbachol for 15 sec. *Ordinate*: response in the presence of EGTA expressed as percent of the contraction observed in its absence (control). A23187, 5×10^{-6} M; carbachol, 1×10^{-5} M. Points are mean $\pm$ SEM for the number of experiments indicated in parentheses.

the sustained phase of an A23187-induced contraction, produces a full, maximal response.

These suggestions for the mechanism of action of A23187 on isolated smooth muscle cells are consistent with current knowledge about the activation of smooth muscle contraction as well as the interaction of A23187 with various biological membranes. The postulate that A23187 produces the initial, EGTA-insensitive contraction by releasing calcium from internal stores gains support from studies which have shown that the ionophore produces calcium efflux from isolated mitochondria (6, 7), from mitochondria within hepatocytes (11), and from vesicles of sarcoplasmic reticulum (8, 9). These observations are particularly relevant, since both mitochondria (21–25) and cytoplasmic vesicles (21, 22, 24–27) have been implicated as possible internal sources of calcium for contraction of smooth muscle. Certain effects of A23187 on other cells also occur in the absence of external calcium and presumably depend on intracellular calcium (28–31). Most of the described effects of A23187 on other systems, however, depend on external calcium (6, 7, 10, 12,

FIG. 6. Effect of calcium on the EGTA-inhibited response of smooth muscle cells to A23187. ●, control (no EGTA); ○, EGTA (3 mM) added 10 sec before addition of A23187 at zero time. Calcium (3 mM) was added at 3 min (arrow) and the cells were fixed 10 sec later. *Ordinate*: contraction expressed as percent of the maximal response of cells to A23187 in each experiment. A23187, 5×10^{-6} M.

13, 32–37), and appear to result from ionophore-mediated transport of calcium across the plasma membrane into the cell (6–12). The sustained, secondary contraction of smooth muscle cells by A23187 appears to result from this action of the ionophore. Further studies with A23187 should aid in establishing the roles of these two calcium pools in contraction of smooth muscle cells.

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