Selectwe previously demonstrated that after several days of serum depriva-ment about one-sixth of confluent cultured canine tracheal myocytes acquire an elongated, structurally and functionally contractile phenotype. These myocytes demonstrated significant shortening on ACh exposure. To evaluate the mechanism by which these myocytes acquire responsiveness to ACh, we assessed receptor-Ca$^{2+}$ coupling using fura 2-AM fluorescence imaging and muscarinic receptor expression using Western analysis. Cells were grown to confluence in 10% fetal bovine serum and then maintained for 7–13 days in serum-free medium. A fraction of serum-deprived cells exhibited reproducible intracellular Ca$^{2+}$ mobilization in response to ACh that was uniformly absent from airway myocytes before serum deprivation. The Ca$^{2+}$ response to 10$^{-4}$ M ACh was ablated by inositol 1,4,5-trisphosphate (IP$_3$) receptor blockade using 10$^{-4}$ M xestospongin C but not by removal of extracellular Ca$^{2+}$. Also, 10$^{-7}$ M atropine or 10$^{-5}$ M 4-diphenylacetoxy-N-methylpiperidine completely blocked the response to ACh, but intracellular Ca$^{2+}$ mobilization was not ablated by 10$^{-6}$ M pirenzepine or 10$^{-5}$ M methoctramine. In contrast, 10$^{-5}$ M bradykinin (BK) was without effect in these ACh-responsive myocytes. Interestingly, myocytes that did not respond to ACh demonstrated robust increases in intracellular Ca$^{2+}$ on exposure to 10$^{-5}$ M BK that were blocked by removal of extracellular Ca$^{2+}$ and were only modestly affected by IP$_3$ receptor blockade. Serum deprivation increased the abundance of M$_3$ receptor protein and of BK$_2$ receptor protein by two- to threefold in whole cell lysates within 2 days of serum deprivation, whereas M$_2$ receptor protein fell by >75%. An increase in M$_3$ receptor abundance and restoration of M$_3$ receptor-mediated Ca$^{2+}$ mobilization occur concomitant with reacquisition of a contractile phenotype during prolonged serum deprivation. These data demonstrate plasticity in muscarinic surface receptor expression and function in a subpopulation of airway myocytes that show mutually exclusive physiological and pharmacological diversity with other cells in the same culture.
and quantified muscarinic and BK receptor abundance using Western analysis.

METHODS

Canine tracheal smooth muscle primary cultures. Tracheae were obtained from adult mongrel dogs, and primary airway smooth muscle cell cultures were established as described previously (2). Briefly, cleaned tracheal muscle was obtained by dissection and minced with scissors. Myocytes were enzymatically dispersed for 60 min at 37°C in buffered saline containing 600 U/ml collagenase, 10 U/ml elastase, and 2 U/ml Nagarse protease. Isolated cells were seeded on uncoated glass coverslips or in uncoated plastic culture plates at a density of 5–10 × 10^3 cells/cm² in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids (NEAA), 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were grown at 37°C in a humidified incubator under 5% CO₂. For passage of cultures at confluence, cells were lifted using 0.05% trypsin and 0.5 mM EDTA and reseeded into three new culture plates per confluent dish. Cells from passage 1 or 2 were used in the studies described.

To induce the contractile phenotype, cultured myocytes were grown to confluence and then serum-containing growth medium was replaced with serum-free Ham’s F-12 medium supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 0.1 mM NEAA, 50 U/ml penicillin, and 50 μg/ml streptomycin. Fresh serum-free medium was provided every 48–72 h thereafter.

Measurement of intracellular free Ca²⁺ responses. To assess calcium responses, cells were incubated in buffered saline, pH 7.4 (in mM: 130 NaCl, 5 KCl, 1 CaCl₂, 1 MgSO₄, 10 HEPES, and 10 dextrose), with 0.1% BSA containing 5 μM fura 2-AM and 50 μM K⁺ fura 2 (50 μM) (17). Calcium responses within a 300 ms at each excitation wavelength and used to calculate calcium concentrations (in nM) at each pixel from an in vitro measurement. A Nikon 20/0.75 objective lens was used, and image size was set to 720 × 540 pixels. At this setting, pixel intensities ranged between 25 and 2,500 gray levels. A fluorescent light excited the cells alternately at 340 and 380 nm with a Lambda 10 filter wheel (Sutter Instruments; Novato, CA). Emitted fluorescence (510 nm) was acquired for 300 ms at each excitation wavelength and used to calculate calcium concentrations (in nM) at each pixel from an in vitro calibration curve of known free Ca²⁺ (0–1.35 μM) and pentapotassium fura 2 (50 μM) (17). Calcium responses within individual cells were determined using Metamorph software (Universal Scientific; Santa Barbara, CA) by circumscribing single myocytes and spatially averaging fura 2 fluorescence within the borders of each cell. Intracellular free Ca²⁺ was recorded for 20 s to establish a baseline and for at least 220 s to characterize peak and plateau responses after addition of either ACh or BK.

Coverslips were placed in a modified Sykes-Moore chamber (with a large glass coverslip forming the bottom of the open chamber) containing 400 μl of BSA-containing HEPES buffer (maximum volume 1 ml). For the addition of agonists, an equal volume (400 μl) of buffer containing twice the target concentration was pipetted into the chamber to ensure rapid mixing for the whole volume. For antagonists, cells were equilibrated with buffer containing the receptor blocker for at least 10 min before addition of agonist.

Intracellular free Ca²⁺ responses to ACh and BK. Two coverslips of tracheal myocytes were prepared as described in Measurement of intracellular free Ca²⁺ responses. Cells were exposed to either ACh (10⁻⁴ M final concentration) or BK (10⁻⁵ M final concentration) in random order, and responses of individual cells were recorded. Myocytes were then washed with three volumes of buffer. After 5 min of equilibration, basal [Ca²⁺]i again was recorded, the other agonist was added to the chamber, and [Ca²⁺]i response was measured. ACh and BK responses were compared in up to 50 individual cells per coverslip.

Assessment of long axis-to-short axis ratio in ACh- and BK-responsive tracheal myocytes. Cells were grown to confluence and serum-deprived as described in Measurement of free Ca²⁺ responses. On day 12 of serum deprivation, myocytes were loaded with fura 2-AM and peak [Ca²⁺]i responses to ACh or BK exposure were recorded as described. Three coverslips of cells were exposed to 10⁻⁷ M BK and 10⁻⁶ M ACh sequentially, and Ca²⁺ mobilization was recorded. Care was taken not to disturb the visual field between agonist exposures as BK was washed out from the chamber. Using Metamorph, we subtracted the baseline 380-nm image from the 380-nm exposure obtained at peak agonist response to yield images that reflect peak calcium responses. Peak responses to BK or to ACh stimulation were pseudocolored green and red, respectively, to allow for identification of BK-responsive, ACh-responsive, or dually responsive myocytes. Using Metamorph software, we measured the long axis and the perpendicular short axis at the widest point (usually at or near the midpoint of the long axis of each cell) of 15 individual ACh-responsive and 15 individual BK-responsive myocytes on each of these three coverslips (45 myocytes per agonist total) and calculated the long axis-to-short axis ratio as an index of cell elongation. Areas of overlap of red and green images appeared yellow when the two peak response images from an individual coverslip were superimposed.

Reproducibility of calcium mobilization to ACh. Coverslips of tracheal myocytes were prepared as described in Measurement of intracellular free Ca²⁺ responses; ACh (10⁻⁴ M final concentration) was added to the chamber and responses of individual cells were recorded. Myocytes that mobilized calcium in response to ACh were noted, and the circumference of each responsive myocyte was demarcated using Metamorph software. Cells were then washed with three volumes of buffer. After 5 min of equilibration, basal [Ca²⁺]i again was recorded. ACh (10⁻⁴ M final concentration) again was added to the chamber, and the [Ca²⁺]i response was measured. First and second responses of individual ACh-responsive myocytes were compared.

Assessment of heterogeneity of [Ca²⁺]i responses to ACh among individual myocytes. Basal (preagonist), peak, and plateau (postagonist) [Ca²⁺]i responses to dual ACh exposures were recorded. Unlike the reproducibility study, two different concentrations of ACh were employed during these measurements. First, myocytes were exposed to ACh at a final concentration ranging from 10⁻¹₂ M to 10⁻⁴ M. The second [Ca²⁺]i response was elicited by exposure to 10⁻⁴ M ACh, to provide a near maximal stimulus. Borders of cells that responded to 10⁻⁴ M ACh were circumscribed, and calcium concentration responses to first and second stimulations were determined. Comparison of the first to the second [Ca²⁺]i responses among individual myocytes revealed the fraction of potentially ACh-responsive myocytes that mobilized intracellular Ca²⁺ on submaximal ACh stimulation.
Effect of muscarinic receptor antagonists on [Ca\(^{2+}\)]\(_i\) response to ACh. Basal and peak [Ca\(^{2+}\)]\(_i\) responses were recorded in individual myocytes stimulated with 10\(^{-6}\) M ACh. Myocytes were washed with two volumes of buffer and then bathed in 400 µl of buffer containing either no antagonist, 10\(^{-5}\) M atropine (nonspecific muscarinic receptor antagonist), 10\(^{-5}\) M pinenepine (M\(_2\) receptor specific), 10\(^{-6}\) M methotrime (M\(_3\) receptor specific), or 10\(^{-6}\) M 4-diphenylacetoxy-N-methylpiperidine (4-DAMP; M\(_3\) receptor specific). After 10 min of equilibration, basal and peak [Ca\(^{2+}\)]\(_i\) levels in response to 10\(^{-4}\) M ACh were again recorded and compared with preantagonist responses.

Calcium pool utilization during Ca\(^{2+}\) mobilization in ACh- and BK-responsive myocytes. Basal and peak [Ca\(^{2+}\)]\(_i\) responses were recorded as described during exposure to 5 mM caffeine. In additional experiments, we assessed the influence of 10\(^{-6}\) M xestospongin C (inositol 1,4,5-trisphosphate (IP\(_3\)) receptor antagonist) or 10\(^{-5}\) M ryanodine (in greater concentration blocks ryanodine receptors) on ACh- or BK-induced calcium mobilization in individual myocytes exposed to 10\(^{-6}\) M ACh or 10\(^{-7}\) M BK before and during incubation with the above agents. Finally, the role of extracellular Ca\(^{2+}\) was explored by recording [Ca\(^{2+}\)]\(_i\) responses to ACh or BK in the presence and then nominal absence of extracellular Ca\(^{2+}\).

Western analysis of receptor protein expression after serum deprivation. Temporal changes in receptor protein composition of canine tracheal myocytes in culture were assessed by Western analysis. After 0–7 days of serum deprivation, cultures were washed with PBS and then total protein homogenates were prepared in extraction buffer [0.3% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 7.6, 0.6 M β-mercaptoethanol, 20 µg/ml leupeptin, 250 µM phenylmethylsulfonyl fluoride, and 50 µg/ml soybean trypsin inhibitor]. Protein from whole cell lysates (15 µg/lane) was size-fractionated by SDS-polyacrylamide gel electrophoresis and protein in TBST with 1% dry milk. Blots were incubated for 1 h at room temperature for 2–4 h in primary antibodies diluted 1:800 for M\(_1\), 1:1,000 for M\(_2\), 1:1,000 for M\(_3\), and 1:1,000 for BK\(_2\) receptor protein. After 0–7 days of serum deprivation, cultures represent the same or different populations. To determine whether the ACh-responsive and BK-responsive myocytes comprise different subpopulations, we quantified calcium responses to both agonists in addition to serum-deprived cultures. The only overlap between BK and ACh responses (seen as yellow in the overlay image of Fig. 2, middle row) reflects divergent responses in physically overlapping myocytes. To evaluate further whether BK or ACh responses occur in different subpopulations, we quantified calcium responses to both agonists in additional 13-day serum-deprived myocytes. As shown in Fig. 3, there is considerable heterogeneity of BK and ACh responses among serum-deprived myocytes. Furthermore, myocytes that exhibit substantial intracellular Ca\(^{2+}\) mobilization in response to ACh exposure have virtually no response to BK (ACh responsive, Fig. 4), and myocytes that exhibit great intracellular Ca\(^{2+}\) elevations during BK exposure have virtually no response to ACh (BK responsive, Fig. 4). Thus ACh-responsive and BK-responsive myocytes comprise different subpopulations of serum-deprived cells. It is also interesting to note that serum-deprived ACh-responsive cells maintained a significantly elevated plateau [Ca\(^{2+}\)]\(_i\) after ACh exposure (Figs. 3 and 4). Not until ACh was washed out of the cell chamber did [Ca\(^{2+}\)]\(_i\) return to baseline. This sustained response is in sharp contrast to serum-deprived BK-responsive myocytes.
which demonstrate a transient peak response to BK without a sustained plateau (Figs. 3 and 4).

To evaluate whether the subpopulations identified above correspond to the elongated (contractile) or non-elongated (noncontractile) phenotype we previously described (2), we quantified the cell shape of ACh- or BK-responsive myocytes shown in Fig. 2. As shown in Fig. 5, the long axis-to-short axis ratio of ACh-responsive myocytes was 2.6-fold greater (\(P = 0.001\), t-test) than that of BK-responsive cells. Thus ACh responsiveness is a property of elongated serum-deprived myocytes, and BK responsiveness occurs in more nonelongated myocytes.

Pharmacological characterization of muscarinic ACh receptors on elongated serum-deprived myocytes. We tested the reproducibility of \([\text{Ca}^{2+}]_{i}\) responses of individual ACh-responsive elongated myocytes to two sequential exposures to \(10^{-4}\) M ACh. Intracellular \([\text{Ca}^{2+}]_{i}\) responses to the second ACh exposure were systematically reduced compared with those observed after the initial ACh exposure, averaging 71.7 \(\pm\) 3.1% of the first response. The two responses were closely correlated \((r^2 = 0.920;\) Fig. 6), and all cells that responded to the first ACh activation exhibited a reduced but clear-cut response to the second ACh exposure. This finding allowed us to evaluate the influence of various muscarinic receptor antagonists on ACh-induced \([\text{Ca}^{2+}]_{i}\) mobilization by administering dual ACh challenges in the absence and presence of muscarinic antagonist. As shown in Table 1, ACh-responsive serum-deprived myocytes demonstrated robust increases in \([\text{Ca}^{2+}]_{i}\) that were completely blocked in a second ACh exposure by \(10^{-6}\) M atropine (nonspecific muscarinic receptor antagonist) or \(10^{-6}\) M 4-DAMP (M3 specific) but were unaffected by \(10^{-6}\) M pirenzepine (M1 specific antagonist). Methoctramine (\(10^{-6}\) M), an M2 receptor antagonist with partial anti-M3 receptor activity, caused partial inhibition of \([\text{Ca}^{2+}]_{i}\) responses to ACh. Thus ACh acts through muscarinic M3 receptors to mobilize intracellular \([\text{Ca}^{2+}]_{i}\) in serum-deprived elongated tracheal myocytes.

Heterogeneous and graded activation of contractile phenotype myocytes by ACh. To evaluate whether individual elongated myocytes in serum-deprived cultures exhibit a graded intracellular \([\text{Ca}^{2+}]_{i}\) response to increasing ACh concentrations, we exposed cells sequentially first to ACh at \(10^{-12}\) M, \(10^{-10}\) M, \(10^{-8}\) M, \(10^{-6}\) M, or \(10^{-4}\) M and second to \(10^{-4}\) M ACh. For each cell that exhibited a clear-cut rise in \([\text{Ca}^{2+}]_{i}\) during the second
ACh stimulation, we determined the rise over baseline [Ca$^{2+}$]$_i$ induced by the first ACh exposure. Because there is no widely accepted standard for the magnitude of [Ca$^{2+}$]$_i$ increase that constitutes a “response,” we calculated the fraction of ACh-responsive myocytes whose peak [Ca$^{2+}$]$_i$ increment over baseline during the first ACh exposure exceeded 100 nM, 200 nM, and 300 nM. Figure 7 demonstrates that 1) maximal stimulation with 10$^{-2}$ M ACh results in a wide range of peak [Ca$^{2+}$]$_i$ elevations in elongated, serum-deprived myocytes; 2) submaximal stimulation with lesser ACh concentrations results in graded peak [Ca$^{2+}$]$_i$ elevations in these cells and there is substantial heterogeneity of response among individual cells; and 3) the fraction of ACh-responsive myocytes that exhibit [Ca$^{2+}$]$_i$ responses increases with ACh concentration whether the threshold for response was set at 100, 200, or 300 nM.

Mechanisms of intracellular Ca$^{2+}$ coupling. Neither ACh-responsive elongated nor BK-responsive nonelongated 13-day serum-deprived myocytes mobilized Ca$^{2+}$ in response to 5 mM caffeine. Also, both subpopulations failed to respond to ryanodine alone, and in the presence of 50 µM ryanodine, second responses to 10$^{-7}$ M BK or 10$^{-8}$ M ACh were unaffected in BK-responsive and ACh-responsive myocytes, respectively (Fig. 8). However, 10 µM xestospongin C, a blocker of IP$_3$ receptors on the sarcoplasmic reticulum (SR), almost completely inhibited Ca$^{2+}$ mobilization in response to a second ACh exposure in ACh-responsive myocytes (Fig. 8). Xestospongin C also partially reduced Ca$^{2+}$ response to a second BK exposure in BK-responsive cells to 50% of that observed during the first exposure to agonist (Fig. 8). The simultaneous presence of ryanodine had no additive effect on the reduction of Ca$^{2+}$ mobilization caused by xestospongin C in either ACh-responsive or BK-responsive myocytes.

Removal of extracellular Ca$^{2+}$ had a profound effect on the second BK response in BK-responsive cells. In the absence of extracellular Ca$^{2+}$, intracellular Ca$^{2+}$ mobilization by BK was completely blocked in serum-deprived cells (Fig. 8). However, removal of extracellular Ca$^{2+}$ did not affect the second peak Ca$^{2+}$ mobilization caused by ACh exposure in serum-deprived ACh-responsive myocytes. Thus initial intracellular Ca$^{2+}$

**Fig. 2.** Individual serum-deprived tracheal myocytes demonstrate exclusive responsiveness to ACh or BK but not both. Three coverslips of cells in disparate states of cell density (a, e, and i) were exposed to 10$^{-7}$ M BK (c, g, and k) and 10$^{-6}$ M ACh (b, f, and j) sequentially, and Ca$^{2+}$ mobilization was noted. Images were created in Metamorph by subtracting the 380-nm image at peak agonist response from baseline. Peak [Ca$^{2+}$]$_i$ mobilization to ACh is artificially displayed in red, whereas peak [Ca$^{2+}$]$_i$ responses to BK are shown in green. ACh and BK peak response images are overlaid in d, h, and l and show essentially no overlap (which would appear as yellow) in ACh or BK responsiveness.
coupling mechanisms that determine peak calcium responses differ importantly in the two subpopulations of serum-deprived myocytes. Elongated myocytes initially mobilize calcium from the SR on ACh stimulation through downstream activation of IP₃ receptors, whereas nonelongated myocytes respond to BK both through initial influx of extracellular Ca²⁺ and an intracellular IP₃ receptor-dependent mechanism.

Western analysis of receptor expression during contractile phenotype modulation. Western analysis of whole culture lysates using anti-muscarinic M₃ receptor antibody revealed a single immunoreactive band of 70 kDa, which was detected in confluent serum-fed (day 0) myocyte cultures but progressively increased in abundance after 2 or 7 days of serum deprivation (Fig. 9). In contrast, M₂ receptor abundance (detected as a 70-kDa immunoreactive band with anti-muscarinic M₂ receptor antibody) decreased significantly with prolonged serum deprivation (Fig. 9). Muscarinic M₁ receptors were not detectable at any time by Western analysis (data not shown). Immunoblots using BK₂ receptor-specific antibody revealed a protein doublet at 42 kDa (Fig. 9). In whole culture lysates, BK₂ receptor body revealed a single immunoreactive band of ~70 kDa, which was detected in confluent serum-fed (day 0) myocyte cultures but progressively increased in abundance after 2 or 7 days of serum deprivation (Fig. 9). In contrast, M₂ receptor abundance (detected as a ~70-kDa immunoreactive band with anti-muscarinic M₂ receptor antibody) decreased significantly with prolonged serum deprivation (Fig. 9). Muscarinic M₁ receptors were not detectable at any time by Western analysis (data not shown). Immunoblots using BK₂ receptor-specific antibody revealed a protein doublet at ~42 kDa (Fig. 9). In whole culture lysates, BK₂ receptor...
abundance increased significantly within 2 days of serum deprivation but tended to decrease somewhat thereafter.

**DISCUSSION**

Cultured airway smooth muscle typically loses many structural and functional characteristics of smooth muscle cells within intact tissue (1, 6, 7, 14, 16). When maintained under growth-promoting conditions, airway myocytes assume a nonelongated spindle shape, contain sparse contractile apparatus proteins, and exhibit virtually no ability to contract. However, we (2) and Ma et al. (11) recently reported that prolonged serum deprivation of confluent cultured airway myocytes promotes a monolayer culture and restores contractile structure and function to a subset of cells. Myocytes that acquire the contractile phenotype accumulate abundant contractile apparatus proteins, exhibit an elongated wormlike morphology, and shorten substantially on electrical field stimulation (11) or ACh exposure (2). The current study was performed to identify mechanisms through which ACh-induced contraction is restored in these contractile cultured cells.

Fig. 6. Reproducibility of [Ca\(^{2+}\)]\(_i\) response to ACh in serum-deprived elongated tracheal myocytes. Peak [Ca\(^{2+}\)]\(_i\) in response to 2 serial 10\(^{-4}\) M ACh exposures are shown. There was reproducible partial reduction of second response vs. first response. On average, the second peak [Ca\(^{2+}\)]\(_i\) was 71.7 ± 3.1% of the first; \(r^2 = 0.920\). All cells that responded to first ACh exposure also responded to second.

We found that ACh stimulates intracellular Ca\(^{2+}\) mobilization within elongated, serum-deprived, contractile airway myocytes but does not do so in serum-fed confluent myocytes or in serum-deprived cells that fail to elongate (Figs. 1–5). Conversely, BK elicited intracellular Ca\(^{2+}\) responses in serum-fed confluent myocytes and in nonelongated serum-deprived cells but not in elongated serum-deprived myocytes. Thus responsiveness to ACh and responsiveness to BK were mutually exclusive in our culture system (Figs. 2 and 4). Pharmacological studies demonstrate clearly that, as in intact trachealis tissue (14), muscarinic M\(_3\) receptors mediate ACh-induced calcium responses in elongated, contractile myocytes (Table 1). Furthermore, increasing ACh concentration, which increases force generation by intact tissue in a graded fashion (13), also increases overall calcium mobilization in cultured contractile airway myocytes both by increasing the number of cells exhibiting any response and by increasing the magnitude of response within individual myocytes (Figs. 6 and 7).

Interestingly, elongated, ACh-responsive, serum-deprived myocytes and nonelongated, BK-responsive cells mobilize calcium in response to agonist by sepa-

**Table 1. Effect of muscarinic receptor antagonists on intracellular Ca\(^{2+}\) mobilization during second 10\(^{-4}\) M ACh exposure**

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Muscarinic Receptor Specificity</th>
<th>Second [Ca(^{2+})](_i) Response to ACh, % of first</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>71.7 ± 3.1</td>
</tr>
<tr>
<td>Atropine</td>
<td>M(_1), M(_2), M(_3)</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Pirenzipine</td>
<td>M(_1)</td>
<td>70.2 ± 10.8</td>
</tr>
<tr>
<td>Methoctramine</td>
<td>M(_2)</td>
<td>29.4 ± 3.4</td>
</tr>
<tr>
<td>4-DAMP</td>
<td>M(_3)</td>
<td>2.5 ± 1.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n = 14–32\) per condition. [Ca\(^{2+}\)]\(_i\), intracellular Ca\(^{2+}\) concentration; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine.
rate mechanisms. The ACh-responsive phenotype mobilizes calcium from a pool that is almost totally inhibited by xestospongin C pretreatment; peak fluorescence from these elongated myocytes during ACh exposure was not affected by removal of extracellular Ca\(^{2+}\) (Fig. 8). Thus calcium from the SR is mobilized in the presence of ACh, and its release is mediated through IP\(_3\) receptors on the SR. These results are in striking contrast with serum-deprived, nonelongated, BK-responsive cells. Xestospongin C only partially inhibited calcium mobilization in BK-responsive cells, whereas removal of extracellular Ca\(^{2+}\) totally inhibited fluorescence in response to agonist (Fig. 8). Thus extracellular Ca\(^{2+}\) may be the major source of the fluorescence response in the BK-responsive subpopulation, and calcium-stimulated calcium release may operate in these cells. Furthermore, the lack of response to caffeine or ryanodine in either subpopulation suggests that canine tracheal myocytes in culture may not express ryanodine receptors on the SR or that this mechanism of calcium release is not predominant in canine tracheal myocytes in culture (Fig. 8).

These findings have several implications. In elongated, contractile myocytes, muscarinic M\(_3\) receptors are found in clusters at the cell surface (2). In contrast, M\(_3\) receptors localize to the perinuclear region in serum-fed confluent or serum-deprived nonelongated airway myocytes (2), neither of which mobilize calcium on ACh exposure (Fig. 1). Thus translocation of muscarinic M\(_3\) receptors to the cell membrane appears to be one mechanism through which M\(_3\) receptor-calcium coupling is restored in elongated myocytes during serum deprivation. It may also be that an increase in total M\(_3\) receptor number during serum deprivation contributes to the restoration of ACh responsiveness (Fig. 9), although the relative importance of each mechanism remains uncertain.

Second, airway smooth muscle cells exhibit plasticity of cell surface receptor expression and function. In intact canine tracheal tissue, ACh but not BK elicits contraction (R. Mitchell, unpublished observations). Culture and passage of canine tracheal myocytes provoke the disappearance of functionally coupled M\(_3\) receptors and the emergence of BK-induced calcium mobilization. Yet, during prolonged serum deprivation, myocytes that reacquire the elongated contractile phenotype also regain the spectrum of agonist responsiveness present within intact tissue. The present study did not address the mechanisms by which M\(_3\) or BK receptor abundance is controlled in individual myocytes. However, the observed increase in total M\(_3\) receptor protein (and accompanying decrease in M\(_2\) receptor protein) suggests that either increased de novo synthesis or diminished degradation of muscarinic M\(_3\) receptors occurs during serum deprivation (Fig. 9). Because the muscarinic M\(_3\) receptor gene promoter has not been cloned, the nuclear mechanisms controlling M\(_3\) receptor gene transcription remain unknown.

Third, elongated, contractile cultured airway myocytes exhibit a wide range of responses to ACh as reflected in their heterogeneous intracellular Ca\(^{2+}\) responses to high concentrations of ACh and their heterogeneous responses to threshold concentrations (Fig. 7). Perhaps this variation among individual elongated myocytes reflects greater or lesser progression toward a "fully" contractile phenotype, but it also resembles the dispersion of other characteristics recently identified among individual myocytes acutely isolated from intact airway muscle (3–5). If this variation among cultured contractile airway myocytes reflects smooth muscle behavior in vivo, then the graded contractile responses to increasing ACh concentration characteristic of intact airway muscle strips (10, 12, 13) may stem both from increasing activation of individual myocytes and from recruitment of increasing numbers of myocytes participating in contraction. To our knowledge, this possibility has not yet been tested directly in intact airway smooth muscle or acutely isolated myocytes.

Previously, Yang and co-workers studied canine tracheal myocytes in culture that had been growth arrested by reduction of serum in the maintenance medium (18, 19, 21). They found that growth arrest...
Ca\(^{2+}\) COUPLING TO M\(_3\) RECEPTORS IN CULTURED AIRWAY MYOCYTES

Increased the number of ligand-binding surface muscarinic receptors (18), that both M\(_2\) and M\(_3\) receptors were present (20), and that pharmacological activation of these receptors increased IP\(_3\) production (19–21). Interestingly, calcium was mobilized in these cultures both by carbachol and by BK in Yang's studies (20–25). Importantly, all cells in culture were treated as a single population rather than as discrete subpopulations of contractile vs. noncontractile (i.e., elongated vs. nonelongated) myocytes. Our studies confirm and extend Yang's work by demonstrating that serum deprivation induces heterogeneity of myocytes in culture and that only the contractile, elongated phenotype demonstrates M\(_3\) receptor-mediated calcium mobilization in response to cholinergic agonist. Thus restoration of ACh-mediated calcium signaling is not a general consequence of serum deprivation. Our data also show that the functional coupling of muscarinic M\(_3\) receptors to calcium mobilization is mediated through IP\(_3\) receptors on the SR in serum-deprived, cultured airway myocytes.

There are limitations to the interpretation of our data. M\(_3\) receptor protein was detected before serum deprivation but was apparently internalized (2) and not functionally coupled to Ca\(^{2+}\) mobilization. Prior studies of cultured confluent human airway myocytes suggest that muscarinic M\(_2\) receptors are associated with the sarcolemma because isoproterenol-induced cAMP formation is inhibited by carbachol and muscarinic M\(_2\) receptor antagonists block this inhibition (16). We also found that methoctramine, an M\(_2\) receptor antagonist, reduced calcium mobilization in response to ACh in elongated serum-deprived myocytes (Table 1); this partial antagonism in part may be due to low-affinity binding of methoctramine to M\(_3\) receptors (20). Furthermore, we could not quantify the distribution of M\(_3\) receptor protein among elongated vs. nonelongated serum-deprived myocytes. Thus it is conceivable that the increase in overall M\(_3\) receptor protein observed during serum deprivation occurred in part within the nonelongated, noncontractile subpopulation. Similarly, we suspect, but cannot prove, that the increase in BK\(_2\) receptor protein evident from analysis of whole culture lysates was localized primarily to the noncontractile myocytes. Also, we could not detect any significant response in either elongated or nonelongated myocytes to caffeine and ryanodine. However, Janssen et al. (8, 9) have noted that freshly dissociated canine tracheal smooth muscle cells rarely contract to these agents, and in only 3 of 7 cells tested (8) was there some transient calcium mobilization in response to concentrations of ryanodine similar to those used in our studies. This finding limits precise interpretation of the lack of a response to caffeine and ryanodine in our serum-deprived myocytes, which thus may indicate a possible continued perturbation in excitation-contraction coupling in the elongated, contractile myocytes. Finally, we cannot be sure that every phenotypically contractile, elongated serum-deprived myocyte does respond to ACh and does not respond to BK. However, we selected visual fields in which both elongated and flattened nonelongated myocytes were present so that we could directly compare [Ca\(^{2+}\)]\(_i\) responses to both ACh and BK. Quantification of cell shape (Fig. 5) confirms that ACh-responsive and BK-responsive subpopulations correspond to the contractile and noncontractile phenotypes we characterized previously (2), and the differences in [Ca\(^{2+}\)]\(_i\) responses and the mechanisms of calcium mobilization to ACh and BK between elongated tracheal myocytes and nonelongated cells in the same image field were striking.

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REFERENCES


