Isolated Guinea Pig Coronary Smooth Muscle Cells

Acetylcholine Induces Hyperpolarization Due to Sarcoplasmic Reticulum Calcium Release Activating Potassium Channels

V. Ganitkevich and G. Isenberg

Smooth muscle cells, dispersed from the circumflex coronary artery of the guinea pig, were studied with the whole-cell configuration of the patch-clamp. The resting potential of about –40 mV was superimposed by spikelike hyperpolarizations (SLHs) up to –20 mV amplitude. The SLHs resulted from spontaneous transient outward currents (spontaneous TOCs) measured under voltage-clamp (~40 or ~50 mV). Acetylcholine (ACH; 10 μM) increased SLHs and TOCs in amplitude and frequency. Atropine blocked the ACh effects. ACh-induced SLHs or TOCs were suppressed by bath application of tetraethylammonium (1 or 10 mM) or by cell dialysis with cesium, suggesting that they result from induction of potassium currents. In cell-attached patches, induction of currents through 130-pS potassium channels was recorded when ACh was bath-applied. An ACh-induced increase in intracellular \([Ca^{2+}]\) is suggested as a second messenger since SLHs and TOCs were suppressed by cell dialysis of 10 mM EGTA. ACh induced SLHs and TOCs in the absence of extracellular calcium. Intracellular application of 5 mg/ml heparin blocked ACh-induced TOCs. When the intracellular calcium stores were depleted by pretreatment with caffeine, the ACh effects were suppressed. Similarly, ACh pretreatment reduced the caffeine-induced outward currents. The results suggested that ACh augments calcium release from the sarcoplasmic reticulum, and the released calcium activates maxi potassium channels. In the single cell, calcium-activated potassium channels generate TOCs and SLHs that sum up to a hyperpolarization of the multicellular tissue. (Circulation Research 1990;67:525–528)

Recent in situ experiments have shown that the large epicardial arteries constrict with cholinergic stimulation.¹ The vasoconstriction was observed in both the presence and absence of endothelium. This finding led to the suggestion that cholinergic mechanisms might be involved in the development of coronary spasm.¹

Vasoconstriction of coronary arteries by acetylcholine (ACH) was reported to occur together with membrane depolarization,² hyperpolarization,³ or constant membrane potential.⁴⁵ This inconsistency of the results may have originated, partially, from the presence or absence of the endothelium in the multicellular tissue. For this reason, we reinvestigated the effect of ACH on membrane potential by using the model of the isolated smooth muscle cell.

In this paper, we report that in smooth muscle cells isolated from the guinea pig coronary artery, ACH consistently hyperpolarized the membrane. For elucidating the underlying mechanism, we studied the effect of ACH on membrane currents, both on whole-cell and single-channel levels, in combination with cell dialysis and drugs interfering with the release of calcium from intracellular calcium stores. The results support the following hypothesis: ACH augments calcium release from the sarcoplasmic reticulum, and the released calcium activates maxi potassium channels. In the single cell, activation of potassium channels generates transient outward currents (TOCs) and spikelike hyperpolarizations (SLHs), which in multicellular tissue may sum up to a smooth hyperpolarization.

Methods

The procedure of cell isolation has been described in detail recently.⁶ The experiments were performed at room temperature (22°C). The superfusing physiological salt solution was composed of (in mM) NaCl

From the Department of Physiology, University of Cologne, Cologne, FRG.

Address for correspondence: Dr. G. Isenberg, Department of Physiology, University of Cologne, Robert-Koch-Strasse 39, D-5000 Cologne, FRG.

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150, KCl 5.4, CaCl₂ 2.5, MgCl₂ 1.2, glucose 20, and HEPES 5, adjusted with NaOH to pH 7.2.

For whole-cell recordings, patch electrodes were filled with an intracellular solution composed of (in mM) KCl 130, Na₂ATP 5, MgCl₂ 2.2, EGTA 0.05, and HEPES 5, adjusted with KOH to pH 7.2. The tips had resistances of 3–5 MΩ and junction potentials of −5 mV. Membrane potentials and currents were recorded with a Brush pen recorder (maximal frequency response, 100 Hz) (Gould Inc., Cleveland).

Bath application of 10 μM ACh required about 5 seconds for a complete change of solution. Not all, but only about 20%, of the viable elongated cells contracted with ACh application. Only these cells are the subject of this study. The nonresponsive cells did contract with caffeine application, and they had normal high-input resistances (>2 GΩ), normal calcium currents, and spontaneous TOCs. The heterogeneity could result from damage of the ACh receptors by the collagenase treatment. Alternatively, ACh receptors may be present in only part of the myocytes of the artery.⁷

Results

The resting potential of the isolated cells was in the range between −30 and −50 mV. On this "baseline," SLHs were superimposed. SLHs had a lifetime of 60 msec on average, and they varied in amplitude (up to 30 mV) and frequency (Figure 1A, before ACh application). The SLHs had their counterpart in spontaneous TOCs (or STOCs⁸) that appeared when the membrane was clamped to −40 or −50 mV. Spontaneous TOCs have been suggested⁹ to result mostly from currents through calcium-activated maxi potassium channels, spontaneous calcium release from intracellular stores delivering activating calcium locally to a small patch of membrane.

When bath application of 10 μM ACh induced a contraction of the cell (monitored as shortening on the TV monitor), the frequency and the amplitude of the SLHs increased (Figure 1A). The ACh-induced SLHs continued to occur as long as ACh was present in the bath. After washout of ACh, the effect disappeared within about 40 seconds.

In the same cell, ACh-induced SLHs could be obtained up to six times. The ACh effect was most prominent during the first ACh application. Figure 1A shows that SLHs of high frequency "fused" with each other in such a way that the baseline of the resting potential became more negative. During the following ACh applications, the effects became attenuated; that is, the augmentation of SLHs was less prominent and the baseline of the potential remained nearly constant (Figure 1B). When the membrane was voltage-clamped to −40 mV, ACh application increased frequency and amplitude of the transient outward currents. Since these TOCs were no longer spontaneous, we call them ACh-induced TOCs.

Evidence for Acetylcholine Increasing Potassium Conductance

In smooth muscle cells isolated from the ear artery, spontaneous TOCs have been reported to be blocked by tetraethylammonium (TEA).⁵ The result was used as argument that spontaneous TOCs flow through TEA-sensitive potassium channels. The spontaneous TOCs of coronary myocytes were completely blocked by superfusion of 10 mM TEA (n=6). Under current clamp conditions, 10 mM TEA abolished spontaneous SLHs and depolarized the membrane (not shown). TEA (10 mM) also blocked the ACh-induced SLHs (Figure 2A). TEA changed the baseline of the membrane potential from −40 mV (ACh-induced hyperpolarization) to −30 mV. The effects were reversed by washout of TEA (Figure 2A). When the effects of
TEA were studied under voltage-clamp conditions, the ACh-induced TOCs were blocked by 10 mM TEA (n=6) and were reduced to less than 10% of the original amplitude by 1 mM TEA (n=4). Additional evidence for TOCs flowing through potassium channels was obtained when the cell was dialyzed with a solution containing no potassium but 145 mM cesium ions. In these cells, investigated at -50 or -40 mV, spontaneous TOCs were absent. Superfusion of 10 µM ACh did not induce TOCs and did not significantly change the current baseline. The absence of a significant reduction of holding current makes it unlikely that ACh has increased the calcium permeability in the cesium-loaded cells.

How Does the Acetylcholine Receptor Couple to the Potassium Channel?

ACh-induced TOCs were reversibly blocked by 10 µM atropine (Figure 2B), suggesting that ACh induces SLHs and TOCs through binding to a muscarinic receptor. In atrial myocytes, muscarinic receptors have been shown to interact with the potassium channel in the membrane phase through a G-protein

FIGURE 3. Bath application of 10 µM activates maxi potassium channels in cell-attached patches. Patch electrodes contained 130 mM potassium. Panel A: Patch potential equals resting potential. No single-channel currents before acetylcholine (ACh) was added, but there are single-channel currents in the presence of ACh. On-line record filtered at 5 Hz. Panel B: Patch potential 20 mV positive to resting potential. On-line record filtered at 20 Hz. Panel C: Computer playback of single-channel currents from tracing in panel B, filtered at 1 kHz.

After bath application of ACh, these currents appeared with increased frequency (Figure 3B). The ACh-induced currents were evaluated by the computer; from the amplitudes at resting potential and at resting potential plus 20 mV, we obtained a slope conductance of 130±20 pS (n=4). Such a value is close to the calcium-activated maxi potassium channel of vascular preparations.

This result, bath application of ACh facilitating the opening of potassium channels isolated in the patch, strongly argues that a cytosolic second messenger couples the occupation of the receptor to the channel activation. The 130-pS conductance suggested that the channel is a calcium-activated potassium channel; hence, we tested the possibility that increments in cytosolic [Ca2+] operate as a second messenger in the ACh-induced opening of potassium channels. With an intracellular solution containing 50 µM EGTA, ACh induced SLHs and TOCs reproducibly. However, dialysis of the cells with a solution containing 10 mM EGTA prevented the ACh-induced TOCs and SLHs as well as the ACh-induced contractions. Thus, the effectiveness of ACh in inducing SLHs or TOCs disappeared if cytosolic [Ca2+] was buffered by EGTA.

Acetylcholine Increases the Potassium Conductance by Augmenting Calcium Release From Intracellular Stores

ACh could have increased cytosolic [Ca2+] by increasing calcium influx. Figure 4A shows that calcium influx is not a necessary requirement. The cell was superfused with calcium free physiological salt solution for 4 minutes, which had reduced the frequency of spontaneous TOCs. Subsequent ACh application largely increased the frequency of TOCs, suggesting that the calcium influx from the extracellular medium cannot be the immediate source for calcium required to activate the potassium channel.

Alternatively, the source of calcium might be an intracellular store from which calcium is released by ACh through a phosphoinositol response. Heparin is a known inhibitor of inositol 1,4,5-trisphosphate binding and inositol trisphosphate–mediated calcium release.

When 5 mg/ml heparin was applied to the cytosol from the patch electrode, the ACh-induced TOCs disappeared (n=4), whereas the spontaneous TOCs remained unmodified (Figure 4D). The result suggests that ACh releases calcium through a phosphoinositol response.

In another set of experiments, we tested whether the ACh-sensitive calcium store overlaps with the caffeine-sensitive one. Bath application of 10 mM caffeine induced a large outward current (Figure 4B), most likely by activating the ryanodine-sensitive calcium-release channel. In unclamped cells, caffeine transiently hyperpolarized cell membrane by 20–30 mV (not shown). Similar to the ACh-induced TOCs, the caffeine-induced current was blocked by extracellular TEA, by intracellular cesium, or by 10 mM intracellular EGTA (not shown), suggesting that the caffeine-induced outward current is carried
through calcium-activated potassium channels. We interpret this to mean that caffeine induced such a large, synchronized calcium release that the TOCs fused together. When ACh was applied shortly after the caffeine removal \((n=5)\), it augmented the TOCs only weakly (Figure 3C). The result suggests that ACh-released calcium stems mostly from a caffeine-sensitive intracellular store.

The interference of ACh- and caffeine-induced calcium release on TOC activity is further demonstrated in Figure 3C. The initial ACh application increased amplitude and frequency of TOCs. In the continuous presence of ACh, application of caffeine further increased the TOC activity, but the resulting outward current was much smaller than the one obtained in the absence of ACh (compare Figure 4, panels B and C). After washout of ACh, the caffeine-induced current completely recovered (not shown). Again, these results support the hypothesis that the ACh effect on potassium currents involves calcium release from a caffeine-sensitive intracellular calcium store.

**Discussion**

The hyperpolarizing effect of ACh on the main coronary artery of the guinea pig by ACh was described by Kitamura and Kuriyama in 1979.3 The present results confirm this result, and the opening of calcium-activated potassium channels is suggested as an underlying mechanism. Coupling from the muscarinic receptor to the opening of potassium channels seems to require an increase in cytosolic \([\text{Ca}^{2+}]\) as a second messenger. Our data present support for the view that if calcium is released from intracellular stores, the messenger ACh receptor is most likely coupled to calcium release through activation of phospholipase C and production of inositol trisphosphate.12

In the isolated cells, the ACh induced SLHs, whereas in multicellular preparations “smooth hyperpolarizations” were found.3 We suggest that cell-to-cell coupling between the many thousands of cells averages (fuses) the SLHs, thereby resulting in a sustained and smooth change in membrane potential.

In conclusion, our results demonstrate that activation of muscarinic ACh receptors hyperpolarizes the membrane of smooth muscle cells from guinea pig coronary arteries. The underlying mechanism is, most likely, calcium release from intracellular stores leading to calcium activation of maxi potassium channels.

**References**


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