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INHIBITION OF CALCIUM TRANSIENTS IN CULTURED VASCULAR SMOOTH MUSCLE CELLS BY PERTUSSIS TOXIN

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SUMMARY: Effects of pertussis toxin on Ca^{2+} transients in rat arterial smooth muscle cells in primary culture were monitored, using quin 2-microfluorometry. In the presence or the absence of extracellular Ca^{2+}, norepinephrine, histamine, caffeine and high extracellular K^+ induced elevations in cytosolic Ca^{2+} concentration. Cytosolic Ca^{2+} elevations induced by norepinephrine and histamine were inhibited by pretreatment of the cells with pertussis toxin, time- and dose-dependently. However, elevations induced by caffeine and K^+-depolarization were unaffected by the pretreatment with this toxin. Thus, it is suggested that GTP binding protein, a pertussis toxin substrate and involved in the receptor-mediated cytosolic Ca^{2+} transients, is not involved in transient elevations in cytosolic Ca^{2+} induced by caffeine and K^+-depolarization in cultured vascular smooth muscle cells. © 1986 Academic Press, Inc.

It is well established that intracellular Ca^{2+} stores play an important role during contraction and relaxation of vascular smooth muscle (1,2). When agonists bind to certain hormone receptors, inositol 1,4,5-trisphosphate (IP_3), a product of the hydrolysis of the plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C, seems to act as a second messenger to release Ca^{2+} from intracellular stores in vascular smooth muscle (3,4). Although IP_3 has messenger function also in so-called non-excitable cells, the underlying mechanism in the generation of IP_3, particularly how the stimuli activate phospholipase

ABBREVIATIONS: $[Ca^{2+}]_i$, intracellular free calcium concentration; EGTA, ethylene glycol-bis (β-aminoethylether) N, N'-tetraacetic acid; HEPES, N-2-Hydroxyethyl-piperazine-N'-2-ethane sulfonic acid; PSS, physiological saline solution; VSMC; vascular smooth muscle cell.

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C, remains unknown (5-7). Recent studies have suggested that receptor-mediated alterations of phospholipid metabolism can be interfered with pertussis toxin in non-excitable cells, thereby indicating the involvement of a GTP binding protein, a substrate for ertussis toxin, in receptor-activation of PIP_2 breakdown (8-10).

In the present study using quin 2-microfluorometry, we obtained the first evidence for involvement of this GTP binding protein in the coupling of receptors to the activation of phospholipase C, resulting in the elevation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat aortic vascular smooth muscle cells (VSMCs) in primary culture. Recently we found that depolarization of plasma membrane, per se, by high extracellular K^+ directly induces a release of Ca^{2+} from intracellular caffeine-sensitive stores (11). The present study also provides evidence that this GTP binding protein is not involved in the release of Ca^{2+} from intracellular stores, as induced by caffeine and K^+-depolarization.

MATERIALS AND METHODS

The materials and their sources were as follows: pertussis toxin (The Chemo-Sero-Therapeutic Research Institute), quin 2/AM (DOTITE), l-norepinephrine hydrochloride (Sigma Chemical Co.), histamine dihydrochloride (WAKO). All other reagents were of the highest grade commercially available.

Cell culture and loading cells with quin 2: Rat aortic medial VSMCs were cultured as described (12) and we used for primary cell cultures, in all cases. On days 5 to 6, just before reaching confluence, the cultured cells on Lux chamber slides were incubated with growth medium containing 50μM quin 2/AM (13) for 60min at 37°C, and then, washed three times with normal physiological saline solution (PSS) at 25°C to remove the dye in the extracellular spaces. The millimolar composition of the normal PSS (pH 7.4 at 25°C) was: NaCl, 135; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 5.5; HEPES, 10. High K^+ solution was prepared by replacing NaCl with KCl, isosmotically. The composition of Ca^{2+}-free PSS was similar to normal PSS, except that it contained 2mM EGTA instead of 1mM $CaCl_2$.

Treatment of VSMCs with pertussis toxin: Prior to loading cells with quin 2 for microfluorometry, VSMCs were incubated with fresh medium containing 20 to 500ng/ml of pertussis toxin for 3 to 24 hours at 37°C. Control cells were exposed to fresh medium, without the pertussis toxin.

Microfluorometry of quin 2: Fluorescence intensity of VSMCs was recorded, using microfluorometry (11,14). Briefly, we used a fluorescence microscope (Zeiss) equipped with a water immersion objective system (Zeiss) and appropriate combination of filters, in which the cells were excited at wavelengths between 350 and 360nm and analyzed at fluorescent wavelengths between 470 and 560nm. Using a pinhole diaphragm in the light axis, the fluorescence intensity in a spot ($<1\mu m^2$) of the cytosol 3μm apart from the nucleus was measured.

Cell viability: High cell viability (>95%) was maintained during the course of each experimental procedure, as assessed by the trypan blue exclusion test (12). Morphological alterations in the VSMCs were nil, as determined by phase contrast microscopy (x400).

RESULTS

When VSMCs were exposed to 10^{-5}M norepinephrine or 10^{-5}M histamine, there was a rapid and transient elevation of $[Ca^{2+}]_i$ (Fig.1A,B). In the presence of extracellular Ca^{2+} (1.0mM Ca^{2+}), norepinephrine and histamine induced transient $[Ca^{2+}]_i$ elevations with peak levels at 2min and 1min, respectively, (the first component), and then $[Ca^{2+}]_i$ declined to a fairly steady level (the second component) within 8min, despite the continuous applications of these agonists. In the absence of extracellular Ca^{2+} (0mM Ca^{2+}, 2mM EGTA), only the first components, (not the second ones) of the

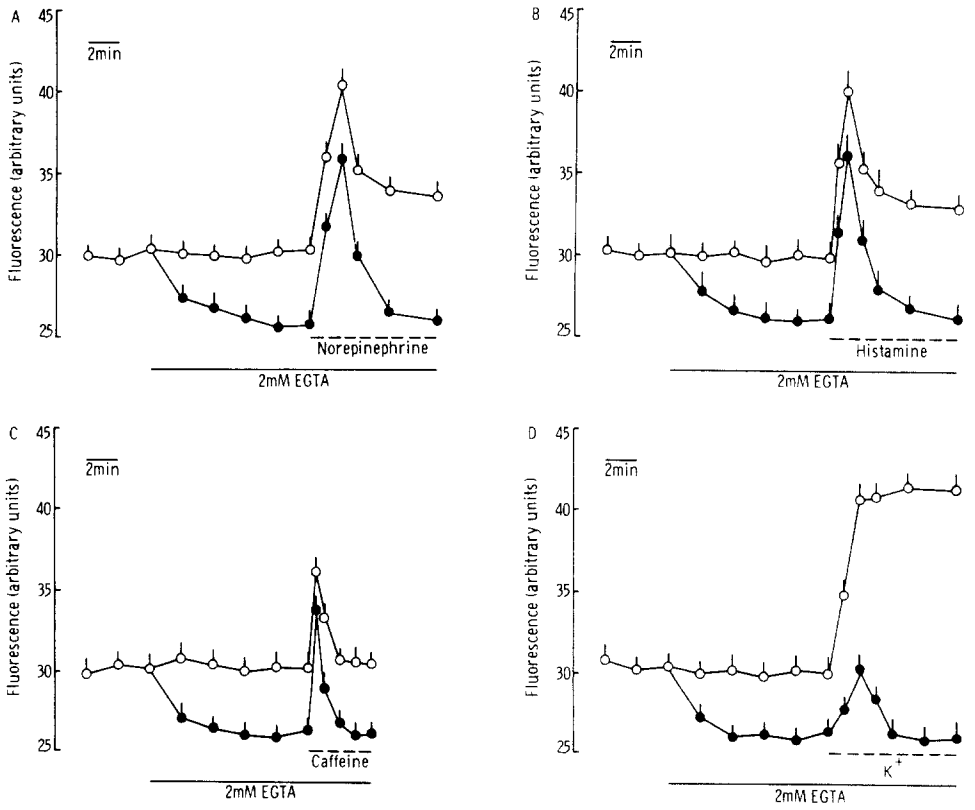

Figure 1. Effects of 10^{-5}M norepinephrine (A), 10^{-5}M histamine (B), 10mM caffeine (C) and 100mM K^+-depolarization (D) (broken lines) on the fluorescence signal from the cytosol in quin 2-loaded VSMCs both in 1.0mM Ca^{2+}-PSS (○) and in Ca^{2+}-free PSS containing 2mM EGTA (●) (solid lines). Data are mean±standard deviations (S.D.) of 4 experiments.

$[Ca^{2+}]_i$ elevations were detected. We have already shown that $[Ca^{2+}]_i$ transients induced by norepinephrine and histamine in cultured rat aortic VSMCs are mediated through the α_1-adrenoceptor and the H_1-histamine receptor, respectively (15,16). Both in the presence and the absence of extracellular Ca^{2+}, 10mM caffeine induced a rapid and transient elevation of $[Ca^{2+}]_i$ with a similar time course and reaching the peak level at 30sec. This suggests that the $[Ca^{2+}]_i$ transient induced by caffeine is also due to a greater extent to the release of Ca^{2+} from intracellular stores (Fig.1C). When VSMCs were exposed to high extracellular K^+ (100mM) in the presence of extracellular Ca^{2+}, there was a rapid elevation of $[Ca^{2+}]_i$, a peak was reached at 2min, and this level was sustained when the VSMCs were incubated in high K^+ solution (Fig.1D). In the absence of extracellular Ca^{2+}, K^+-depolarization also induced a transient elevation of $[Ca^{2+}]_i$, which reached a peak level at 2min, and then, declined to a pre-depolarization level at 4min. The extent of the observed elevation of $[Ca^{2+}]_i$ induced by K^+-depolarization in the absence of extracellular Ca^{2+} was much less than that observed in the presence of extracellular Ca^{2+}. These results suggest that with K^+-depolarization, Ca^{2+} can be directly released from intracellular stores, and that the marked and sustained elevation of $[Ca^{2+}]_i$ observed in the presence of extracellular Ca^{2+} is mainly due to influx of extracellular Ca^{2+} (14).

The pretreatment of VSMCs with 500ng/ml pertussis toxin reduced the first component elevation (at 2min) of $[Ca^{2+}]_i$ induced by 10^{-5}M norepinephrine. The extent of the reduction of $[Ca^{2+}]_i$ elevation was dependent on time of incubation with the toxin, both in the presence and the absence of extracellular Ca^{2+} (Fig.2). This inhibition of the first component elevation of $[Ca^{2+}]_i$ was evident after 12hrs and was almost complete at 24hrs ($p<0.05$ by an analysis of variance). The higher was the amount of pertussis toxin used to pretreat VSMCs for 24hrs, the higher was the extent of inhibition of the first component elevation of $[Ca^{2+}]_i$ induced by 10^{-5}M norepinephrine, both in the presence and the absence of

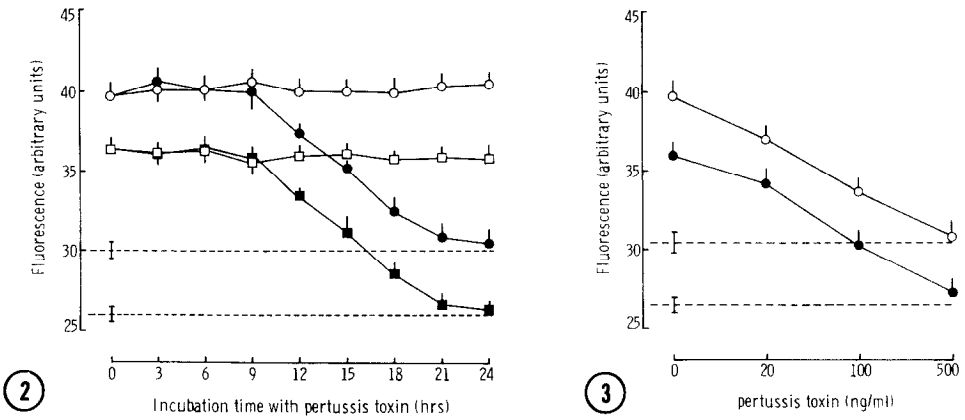

Figure 2. Time-dependent effects of the pretreatment of VSMCs with 500ng/ml pertussis toxin on 10^{-5}M norepinephrine-induced transient rise in fluorescence signal (the first component); toxin treated cells (●, ■) and control cells (○, □) both in 1.0mM Ca^{2+}-PSS (○, ●) and in Ca^{2+}-free PSS containing 2mM EGTA (□, ■). The upper broken line indicates the pre-exposure level of fluorescence to norepinephrine in 1.0mM Ca^{2+}-PSS and the lower one indicates the pre-exposure level of fluorescence to norepinephrine in Ca^{2+}-free PSS containing 2mM EGTA. Data are mean±S.D. of 4 experiments.

Figure 3. Effects of the pretreatment of VSMCs with various doses of pertussis toxin on 10^{-5}M norepinephrine-induced transient rise in fluorescence signal (the first component) both in 1.0mM Ca^{2+}-PSS (○) and in Ca^{2+}-free PSS containing 2mM EGTA (●). The upper broken line indicates the pre-exposure level of fluorescence to norepinephrine in 1.0mM Ca^{2+}-PSS and the lower one indicates the pre-exposure level of fluorescence to norepinephrine in Ca^{2+}-free PSS containing 2mM EGTA. Data are mean±S.D. of 4 experiments.

extracellular Ca^{2+} (Fig.3). Thus, inhibition of the norepinephrine-induced elevation of $[Ca^{2+}]_i$ by pertussis toxin was also dose-dependent ($p<0.05$ by an analysis of variance). Fig.3 also shows that pretreatment with 500ng/ml pertussis toxin completely inhibited $[Ca^{2+}]_i$ elevation induced by 10^{-5}M norepinephrine. The effects of the pretreatment of VSMCs with 500ng/ml pertussis toxin for 24 hours on the peak elevation of $[Ca^{2+}]_i$ induced by 10^{-5}M norepinephrine, 10^{-5}M histamine, 10mM caffeine and 100mM K^+-depolarization, both in the presence and the absence of extracellular Ca^{2+}, are shown in Fig.4A and B. Not only the first components, but also the second components of the $[Ca^{2+}]_i$ transients induced by norepinephrine and histamine were almost completely inhibited. On the other hand, peak elevations of $[Ca^{2+}]_i$ induced by caffeine and K^+-depolarization, measured at 30sec and 2min after stimulation, respectively, were unaffected by

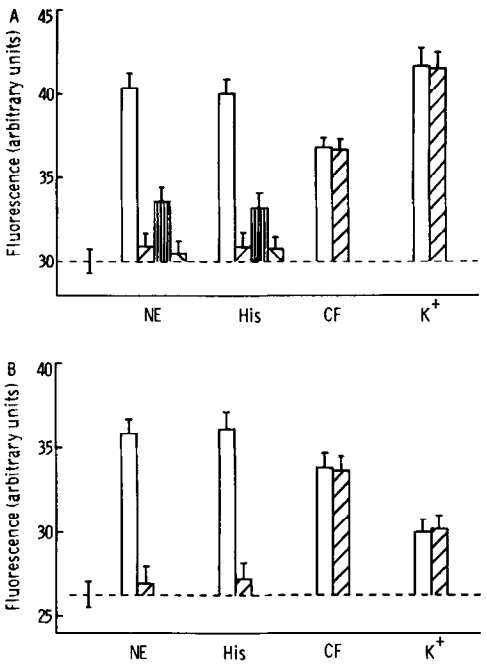

Figure 4. Effects of the pretreatment of VSMCs with 500ng/ml pertussis toxin for 24 hours on the elevations of fluorescence signal induced by 10^{-5}M norepinephrine, 10^{-5}M histamine, 10mM caffeine and 100mM K^+-depolarization both in 1.0mM Ca^{2+}-PSS (A) and in Ca^{2+}-free PSS containing 2mM EGTA (B). The first components of norepinephrine-and histamine-induced transient rise of fluorescence signals (measured at 2min and 1min of exposure to norepinephrine and histamine, respectively) in control cells (□) and in toxin treated cells (▨), and the second components of them (measured at 8min exposure to both norepinephrine and histamine) in control cells (■) and in toxin treated cells (▩). Caffeine- and K^+-depolarization-induced elevations of fluorescence signal (measured at 30sec and 2min after exposure to caffeine and high K^+, respectively) in control cells (□) and in toxin treated cells (▨). Broken lines in (A) and (B) indicate the pre-exposure levels of fluorescence of VSMCs in 1.0mM Ca^{2+}-PSS and in Ca^{2+}-free PSS containing 2mM EGTA, respectively. Data are mean±S.D. of 4 experiments. NE: norepinephrine, His: histamine, CF: caffeine, K^+: K^+-depolarization.

pretreatment with pertussis toxin, both in the presence and the absence of extracellular Ca^{2+}.

DISCUSSION

Previous studies indicated that a pertussis toxin-specific regulatory protein, a GTP binding protein, coupled receptor-stimulation to cellular Ca^{2+} mobilization in various cell types (8-10). Although Ca^{2+} released from intracellular stores has been implicated as the primary regulator of contractile activity of VSMCs (1,2), as yet very little, if any, information is available on the effect of pertussis toxin on this GTP

binding protein which may be associated with the agonists-stimulated contraction of VSMCs. The present study showed that a fairly high concentration (500ng/ml) of the toxin with a long period of incubation (24hrs at 37°C) was required for the complete inhibition of a GTP binding protein in VSMCs, as was noted in the case of cardiac muscle cells (17,18). From this point of view, cultured cells may be more suitable than strips of blood vessels or dispersed muscle cells to study the effect of this toxin on VSMCs.

Since norepinephrine induced transient elevations of $[Ca^{2+}]_i$ (the first component) with a similar time course and extent, both in the presence and the absence of extracellular Ca^{2+}, this component could be due to the receptor-mediated release of Ca^{2+} from intracellular stores (15). The events were the same with histamine (16). Agonists binding to receptors led to generation of the second messenger, IP_3, from PIP_2 by the activation of phospholipase C in vascular smooth muscle (19-21). In the present study, the first component elevations of $[Ca^{2+}]_i$ induced by norepinephrine or histamine were completely inhibited by pretreating VSMCs with pertussis toxin. Thus, it is suggested that involvement of a pertussis toxin-specific GTP binding protein is a prerequisite for the early stage production of the second messenger in VSMCs. It is not plausible that an inhibition of $[Ca^{2+}]_i$ transient is due to the direct interaction of pertussis toxin, per se, with receptors, because pertussis toxin apparently does not moderate the binding properties of membrane receptors (9). Both norepinephrine and histamine induced the second component elevation of $[Ca^{2+}]_i$ only in the presence of extracellular Ca^{2+}, thereby suggesting that this elevation of $[Ca^{2+}]_i$ is due to the influx of extracellular Ca^{2+} through receptor-operated Ca^{2+} channels (15,16). In the present study, pertussis toxin completely inhibited the second component $[Ca^{2+}]_i$ elevation induced by norepinephrine or histamine, hence, this toxin-specific GTP binding protein is probably involved in the signaling system related to generation of the second messenger to the Ca^{2+} channel.

We reported that K^+-depolarization can directly release Ca^{2+} from intracellular stores and, thus, induce a transient elevation of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, and that there may be a almost complete overlap of caffeine- and K^+-depolarization-sensitive intracellular Ca^{2+} stores in cultured VSMCs (11). The possible second messenger of K^+-depolarization-induced Ca^{2+} release from intracellular stores has been debated: in skeletal muscle, IP_3 was suggested to be the second messenger of K^+-depolarization-induced Ca^{2+} release (22-24), and in VSMCs, however, it is suggested that K^+-depolarization does not induce the breakdown of PIP_2 to IP_3 (25). The present study demonstrates that both the caffeine- and K^+-depolarization-induced Ca^{2+} release from intracellular stores is unaffected by pretreatment with pertussis toxin. This suggests that a pertussis toxin-specific GTP binding protein in the signaling system for generating the second messenger is not involved in release of Ca^{2+} from intracellular stores, as induced by caffeine or K^+-depolarization in VSMCs. In conclusion, our study suggests that a pertussis toxin-specific GTP binding protein couples the α_1-adrenoceptor (15) and H_1-histamine receptor (16) to the activation of phospholipase C for generating IP_3 and that this protein is not involved in the caffeine- and K^+-depolarization-induced $[Ca^{2+}]_i$ elevation in cultured rat aortic VSMCs.

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