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A Patch-Clamp Study of the Ca^{2+} Mobilization from Internal Stores in Bovine Aortic Endothelial Cells. II. Effects of Thapsigargin on the Cellular Ca^{2+} Homeostasis

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Summary. Evidence was provided, in the preceding paper (Thuringer & Sauvé, 1992), that the external Ca^{2+}-dependent phase of the Ca^{2+} signals evoked by bradykinin (BK) or caffeine in bovine aortic endothelial cells (BAE), differ in their respective sensitivity to procaine. To examine whether the emptying of the $InsP_3$-sensitive Ca^{2+} store is the signal for activating the agonist-evoked Ca^{2+} entry, we have investigated the effects of thapsigargin (TSG), a known inhibitor of the microsomal Ca^{2+}-ATPase activity in a variety of cell types, via the activity of calcium-activated potassium channels [K(Ca^{2+}) channels]. In cell-attached experiments, the external application of TSG caused a sustained or oscillatory activation of K(Ca^{2+}) channels depending on both the cells and doses tested. The TSG-evoked channel activity could be reversibly blocked by removing extracellular Ca^{2+}, and strongly decreased by adding 10 mM procaine to the bath medium. In Ca^{2+}-free external conditions, TSG did not promote an apparent Ca^{2+} discharge from internal stores but prevented in a dose- and time-dependent manner the subsequent agonist-evoked channel activity related to the release of internally sequestered Ca^{2+}. These results confirm that TSG and BK release Ca^{2+} from the same internal stores but with different kinetics. Because the channel response to caffeine was found to be poorly sensitive to procaine, in contrast to that evoked by BK and TSG, it may be concluded that both BK and TSG activate the same Ca^{2+} entry pathway. Therefore, the emptying of the $InsP_3$-sensitive Ca^{2+} store is likely to be the main signal for activating the agonist-evoked Ca^{2+} entry in BAE cells.

Key Words procaine · bradykinin · calcium-activated potassium channels

Introduction

It is now well established that the acute hydrolysis of phosphatidylinositol-4,5-bisphosphate with release of the soluble messenger inositol-1,4,5-triphosphate ($InsP_3$), initiates the mobilization of internally sequestered Ca^{2+} in BAE cells treated with 10 nM bradykinin (BK) (Pollock, Wreggett & Irvine, 1988; Freay et al., 1989; Myers & Larkins, 1989). The resulting Ca^{2+} release from intracellular stores is followed by a long-lasting entry of Ca^{2+} from the extracellular space through a cell pathway which is, as yet, poorly defined (Colden-Stanfield et al., 1987, 1990; Schilling et al., 1988; Adams et al., 1989; Schilling, Rajan & Strobl-Jager, 1989; Sauvé et al., 1990; Thuringer, Diarra & Sauvé, 1991). Electrophysiological studies have provided strong evidence that the agonist-evoked Ca^{2+} entry is not mediated by a classical voltage-sensitive Ca^{2+} channel (Colden-Stanfield et al., 1987; Johns et al., 1987; Takeda, Schini & Stoeckel, 1987; Cannell & Sage, 1989) but conclusive results concerning the existence of a Ca^{2+} permeable channel controlled by BK are still required. Among the different mechanisms proposed for the regulation of agonist-stimulated Ca^{2+} entry, one proposal involves a pathway controlled by the fullness state of intracellular Ca^{2+} stores, as first assumed for rat parotid acinar cells (Putney, 1986; Merritt & Rink, 1987; Takemura & Putney, 1989). According to this mechanism, termed capacitative Ca^{2+} entry, the Ca^{2+} influx across the plasma membrane is secondarily activated by emptying internal Ca^{2+} stores. This may account for the phenomenon of receptor-activated divalent cation entry reported in stimulated endothelial cells despite the removal of the agonist (Hallam, Jacob & Merritt, 1989; Jacob, 1990; Lückhoff & Busse, 1990; Thuringer et al., 1991).

This model has been recently confirmed in a variety of nonexcitable cell types by investigating the action of a sesquiterpene lactone, thapsigargin (TSG) (Rasmussen, Christensen & Sandberg, 1978), known to release internally sequestered Ca^{2+} by a mechanism independent of the receptor stimulation and $InsP_3$ formation (Scharff et al., 1988; Foder, Scharff & Thastrup, 1989; Takemura et al., 1989; Thastrup et al., 1989, 1990; Kwan et al., 1990; Thastrup, 1990; Foskett, Roifman & Wong, 1991;

Sarkadi et al., 1991; Dolor et al., 1992). This TSG-induced Ca^{2+} release is followed by a sustained Ca^{2+} influx from the extracellular space, using the same pathway as that activated by agonists (Takemura et al., 1989; Kwan et al., 1990; Foskett et al., 1991; Dolor et al., 1992), consistent with the concept of capacitative Ca^{2+} entry in these cell types. Furthermore, studies on rat liver microsomes have shown that TSG discharges Ca^{2+} from nonmitochondrial stores through unknown pathways, and prevents their subsequent loading by specific inhibition of the membrane Ca^{2+}-ATPase of endoplasmic reticulum (Thastrup et al., 1990).

Evidence was provided in the preceding paper (Thuringer & Sauvé, 1992) for the existence of functionally distinguishable $InsP_3$-sensitive and caffeine-sensitive Ca^{2+} stores in BAE cells. In addition, we found that the Ca^{2+} influx evoked by BK or caffeine differs in their respective sensitivity to procaine. Because some spontaneous or caffeine-induced oscillations in $[Ca^{2+}]i$ have been recently reported in TSG-treated acinar cells (Foskett et al., 1991) and in view of the fact that BK and caffeine seem to initiate different Ca^{2+} entry pathways, we have examined the effects of thapsigargin in our cell preparation. By recording the activity of $K(Ca^{2+})$ channels in cell-attached patches of confluent BAE cells, we found that the cell exposure to TSG dose-dependently induced a channel activation process which could adopt an oscillatory behavior in some cells. Moreover, we observed that procaine inhibits the Ca^{2+} entry elicited by TSG. The significance of our findings in terms of the mechanisms underlying the regulation of the Ca^{2+} influx and the spatial distribution of internally sequestered Ca^{2+} is discussed.

Abbreviations

BAE cell: bovine aortic endothelial cell; BK: bradykinin; TSG: thapsigargin; $InsP_3$: inositol 1,4,5-triphosphate; EGTA: ethylene glycol-bis(*b*-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; HEPES: N-2-hydroxyethytlpiperazine-N′-2-ethanesulfonic acid; DMSO: dimethyl sulfoxide; $[Ca^{2+}]i$: cytosolic free calcium ion concentration; $K(Ca^{2+})$ channel: calcium-activated potassium channel; iK1 channel: inward-rectifying potassium channel; Vp: potential applied in the patch pipette.

Materials and Methods

All reagents were obtained from Sigma (St. Louis, MO), except TSG which was purchased from LC Services (Woburn, MA). The standard bath solution was Earle medium containing (in mM): 121.3 NaCl, 6.0 $NaHCO_3$, 1.0 NaH_2PO_4, 5.4 KCl, 0.8 $MgSO_4$, 1.8 $CaCl_2$, 5.5 glucose, 10 NaOH and 25 HEPES (pH 7.3). The Ca^{2+}-free bath solution was prepared by adding 2 mM EGTA with no $CaCl_2$. BK and TSG were dissolved as stocks at 10 μM in H_2O and 10 mM in DMSO, respectively. Procaine was added to Earle medium at the final concentration of 10 mM and the pH of solutions was checked just before used. The TSG solutions were covered with aluminium foil to protect from light, and used within 3 hr of preparation. The pipette solution contained (in mM): 200 KCl, 0.5 $MgSO_4$, 0.91 $CaCl_2$, 1 EGTA, 25 HEPES and 10 KOH (pH 7.3) for a Ca^{2+} concentration of 1 μM.

BAE cells of confluent monolayers were prepared as described in the preceding paper (Thuringer & Sauvé, 1992). Membrane current recordings were performed in the cell-attached patch-clamp configuration (Hamill et al., 1981) and the potential values reported (Vp) corresponded to the potential applied in the patch pipette as described before (Thuringer & Sauvé, 1992). The electrical recordings were carried out in a continuous perfusion mode with a time delay of 5 sec to fully exchange the external medium. All experiments were carried out at room temperature (usually 20°–23° C).

Results

Effects of TSG on the Single-Channel Activity of BAE Cells

To record single-channel currents mainly carried by K^+ ions entering the cell, a potential (Vp) of +30 mV was applied to the pipette containing a high K^+ solution in cell-attached patches of BAE cells in confluent monolayers. The activity of voltage-insensitive $K(Ca^{2+})$ channels, linked to a rise of cytosolic free Ca^{2+} level, was rarely observed in nonstimulated BAE cells, as shown in the preceding paper (Thuringer & Sauvé, 1992). No additional channel activity could be induced over a 200-sec period by exposing cells to TSG at concentrations lower than 2 μM at room temperature. This concentration of TSG which is considered as the maximally effective dose in other cell preparations (Takemura et al., 1989; Kwan et al., 1990; Foskett et al., 1991; Sarkadi et al., 1991), was barely capable of promoting a sufficient increase in $[Ca^{2+}]i$ to activate $K(Ca^{2+})$ channels (n = 15 cells). In 60% of the patches, openings of $K(Ca^{2+})$ channels during the external application of 2 μM TSG, lasted about 100 sec and this single-channel activity was seldom observed in the following period (*data not shown*). Higher concentrations of TSG (7–10 μM) invariably led to a more sustained activation of $K(Ca^{2+})$ channels in nonstimulated BAE cells (Fig. 1*A*) or after cell stimulation by 10 nM BK (Fig. 1*B*). There was a time delay ranging from 60 to 80 sec between the cell exposure to TSG and the appearance of single-channel events whereas the mean value of such a delay is 33 sec in BK-stimulated cells (Sauvé et al., 1990). This delayed channel activation could be consecutive to a slower increase in $[Ca^{2+}]i$ induced by TSG compared to that induced by

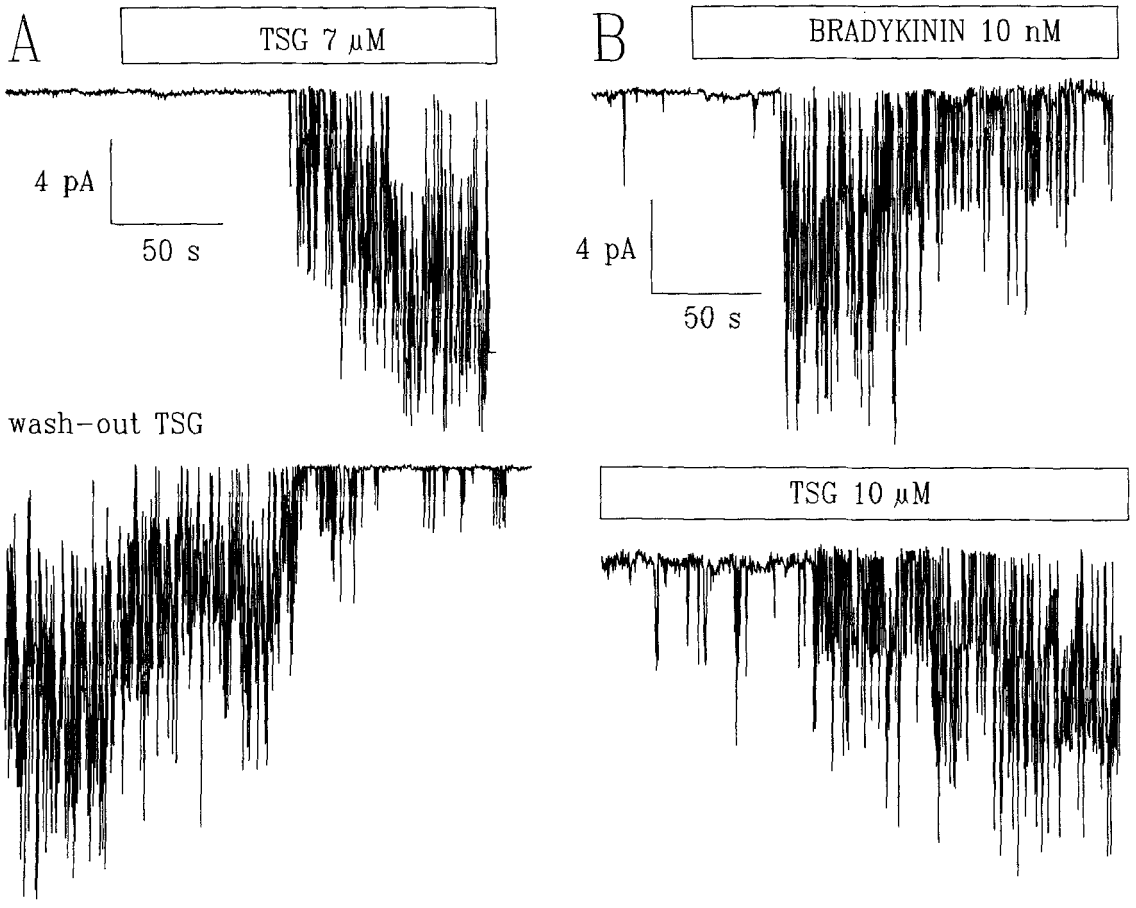

Fig. 1. Typical single-channel responses induced by external thapsigargin (TSG). Continuous recordings of inward K^+ currents flowing through $K(Ca^{2+})$ channels activated by TSG in two different cell-attached patches on BAE cells of confluent monolayers, in non-stimulated cell (*A*) and after (*B*) the cell stimulation by 10 nM bradykinin. A potential of +30 mV was applied to the patch pipette containing a high K^+ solution. Recordings were performed in the continuous perfusion mode of normal Earle solution supplemented with TSG or BK, as indicated on the top of each trace. The time constant of solution change at the onset of a drug application was estimated to be 5 sec. All experiments were performed at room temperature (usually 20°–23° C). Each trace contains 1500 pts of the original current record.

agonists, as already reported in other cell types (Thastrup, 1990). In addition, this lag could be partially related to a low membrane permeation of TSG at room temperature (20°–23° C). In contrast to the sustained elevation of $[Ca^{2+}]i$ initiated by lower doses of TSG in other cell preparations (Thastrup et al., 1989; Thastrup, 1990; Foskett et al., 1991; Dolor et al., 1992), a slow disappearance of the TSG-induced channel activity was observed by removing the agent from the bath medium (Fig. 1*A*). After a long period of cell superfusion with the standard bath solution, $K(Ca^{2+})$ channels could be reactivated in response to a subsequent application of BK or TSG (*data not shown*). It must be noticed that the number of reactivable channels and, thus, the amplitude of the subsequent single-channel response to TSG or agonist, decreased slowly with time of recording.

Dose-Dependency of the TSG Effects

TSG (7–10 μM) provokes a marked activation of the $K(Ca^{2+})$ channels in all the cells tested, with, however, two different profiles: a sustained activity over recording periods as long as 8 min (n = 20), or some brief oscillations (n = 3) characterized by single-channel bursts of 60-sec duration separated by similar silent periods (60–70 sec) as shown in Fig. 2 (upper trace). Some TSG-induced oscillations in $[Ca^{2+}]i$ have been recently reported in parotid acinar cells and did not involve a repetitive release of sequestered Ca^{2+} from the $InsP_3$-sensitive store (Foskett et al., 1991). These oscillations could be induced by caffeine (1–20 mM) in nonoscillating TSG-treated acinar cells which required the continued presence of caffeine (Foskett & Wong, 1991). In vascular endothelial cells, oscillations in $[Ca^{2+}]i$

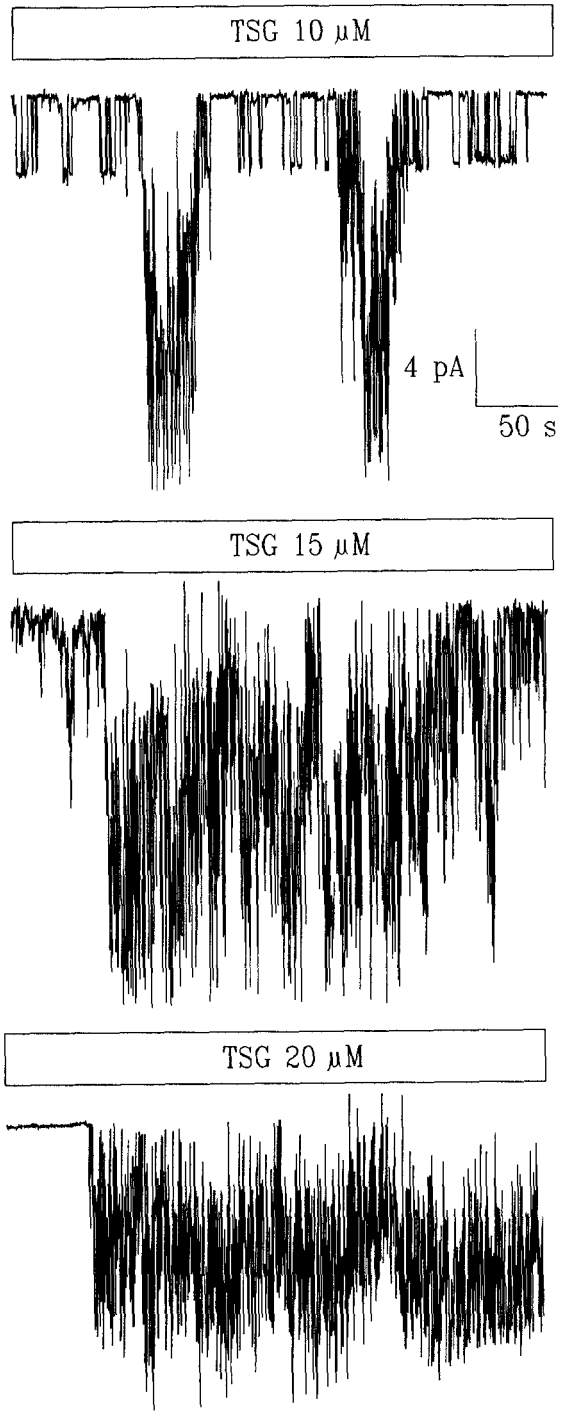

Fig. 2. Dose dependency of the TSG-induced single-channel response. $K(Ca^{2+})$ channel activity recorded upon TSG application at various concentrations in three different cell-attached patches on cells of confluent monolayers. Each dose tested was applied at the beginning of the current trace, as indicated (bath exchange within 5 sec). Note small unitary currents flowing through an unknown channel at the beginning of the middle trace. Pipette filled with a high K^+ solution and Vp of +30 mV (temperature 20°–23° C). Each trace contains 2000 pts of the original current record.

occurred in response to BK application onto TSG-pretreated monolayers (Laskey et al., 1991). However, the addition of caffeine (1 mM) or BK (10 nM) to the bath medium containing 7–10 μM TSG was without effect on the sustained $K(Ca^{2+})$ channel activity recorded in our cell preparation (*data not shown*). Because of some variability among cells in their single-channel responses recorded, we have examined the effects of higher TSG concentrations in order to determine the maximally effective dose in BAE cells. Increasing the dose to 15 μM prolonged systematically the cell response, and in some cells (Fig. 2, middle trace), brief oscillations of the $K(Ca^{2+})$ channel activity seemed to be superimposed upon a slowly fluctuating level of activity. A sustained activation process of the $K(Ca^{2+})$ channels could be recorded at 20 μM TSG, and did not require the continued presence of the agent (Fig. 2, lower trace). Therefore, 20 μM appears to represent the maximally effective dose causing, in BAE cells, an irreversible increase in $[Ca^{2+}]i$ comparable to that produced by TSG at nanomolar concentrations in other cell types (Thastrup, 1990). Independently of the doses tested, the time delay between the cell exposure to TSG and the appearance of the first single-channel events, ranged from 60 to 80 sec (n = 40) and rarely from 100 to 160 sec (n = 5).

Ability to Release Ca^{2+} from Intracellular Stores

Subsequent experiments were carried out to test the ability of TSG to interact with internal Ca^{2+} stores of BAE cells (Fig. 3). Figure 3*A* shows the channel response of confluent BAE cells in conditions where the cells were stimulated first by 10 nM BK then by 7 μM TSG in Ca^{2+}-free conditions. As previously reported (Thuringer et al., 1991), the cell exposure to BK in absence of external calcium caused a transient activation of $K(Ca^{2+})$ channels which reflected Ca^{2+} release from internal stores coupled to an internal Ca^{2+} reuptake and/or extrusion from the cell. Following the removal of BK, no additional single-channel activity was triggered over a 100-sec period by the subsequent addition of TSG into the Ca^{2+}-free solution. The readmission of calcium into the bath medium produced a fast and transient reactivation of the channels present in the patch area (*data not shown*), as expected for an agonist-evoked Ca^{2+} influx (Thuringer et al., 1991). This protocol was repeated by adding TSG before BK into the Ca^{2+}-free bath solution. As shown in Fig. 3*B*, no $K(Ca^{2+})$ channel activity was induced by the cell application of TSG. In addition, a decrease in the unitary amplitude of iK1 channel current was observed due to a

cell depolarization in Ca^{2+}-free conditions, suggesting an inhibitory effect of this medium on active $K(Ca^{2+})$ channels present outside the membrane patch area (Sauvé et al., 1990). The subsequent addition of 10 nM BK produced a typical activation of $K(Ca^{2+})$ channels superimposed to the transient increase in the unitary current amplitude of iK1 channel (Sauvé et al., 1990). Except for two different cell-attached patches where a brief activation of a few channels could be detected, the majority of patches remained silent upon an initial cell exposure to TSG (7, 10, 20 μM) in Ca^{2+}-free external conditions (n = 20). However, a higher dose of TSG (20 μM) externally applied in Ca^{2+}-free conditions (n = 5) invariably prevented the $K(Ca^{2+})$ channel activity normally induced by the agonist in absence of external calcium. Figure 3*C* clearly shows that the agonist lost its ability to induce an internal Ca^{2+} mobilization after a 120-sec period of cell exposure to TSG 20 μM with no external Ca^{2+}. Therefore, a TSG treatment of BAE cells blocked gradually, in a dose- and time-dependent manner, the subsequent release of intracellular Ca^{2+} from at least an $InsP_3$-releasable Ca^{2+} store. These results are confirmed in subsequent experiments where the ability of 10 nM BK to discharge Ca^{2+} from internal stores was tested on a cell which showed a sustained activity of $K(Ca^{2+})$ channels in response to the external application of 10 μM TSG (Fig. 4). The removal of external Ca^{2+} suppressed entirely the $K(Ca^{2+})$ channel activity evoked by TSG, in accordance with previous results reported for the TSG-induced rise in $[Ca^{2+}]i$ in other cell preparations. Concomittant to the disappearance of $K(Ca^{2+})$ channel openings, a reduction in unitary amplitude of the iK1 channel current occurred indicating that the effective potential across the membrane patch area decreased due to cell depolarization. The subsequent application of BK neither elicited a $K(Ca^{2+})$ channel activity nor the transient hyperpolarization usually observed in BAE cells (Sauvé et al., 1990). The sustained activity of $K(Ca^{2+})$ channels could be again evoked by TSG upon the readmission of calcium to the bath medium.

These results strongly suggest that TSG induced a Ca^{2+} discharge from internal Ca^{2+} stores including the $InsP_3$-sensitive store. The unexpected observa-

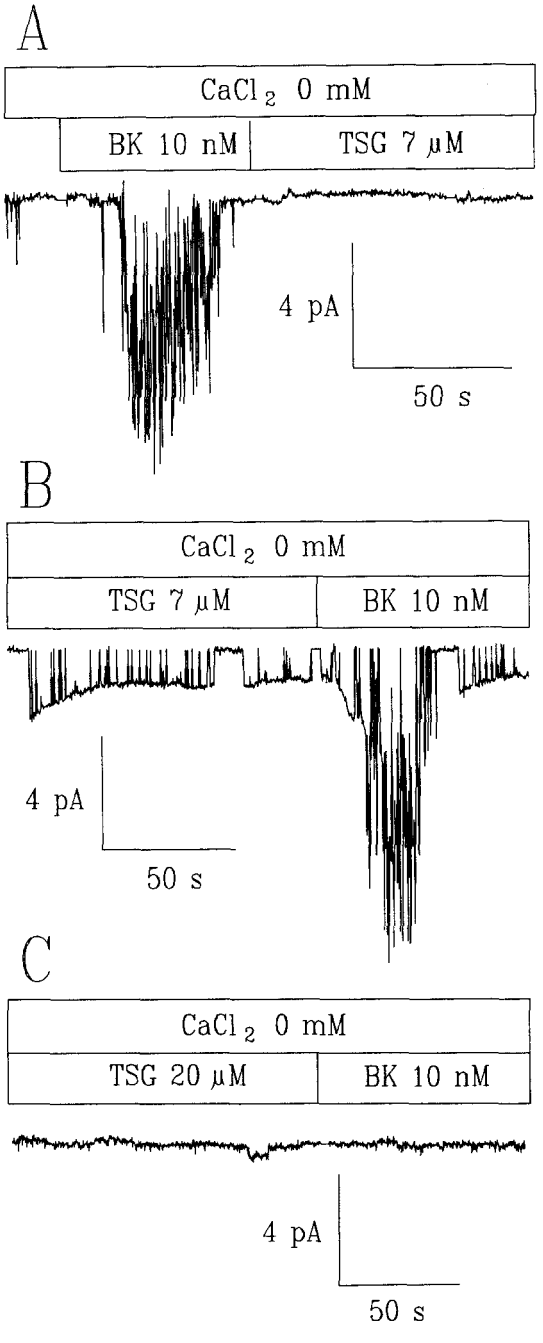

Fig. 3. Contribution of external calcium to the TSG-induced activation process of $K(Ca^{2+})$ channels. Continuous perfusion experiments carried out on three different cells in the cell-attached configuration at a constant Vp +30 mV and with a high K^+ pipette solution. The emptying state of intracellular Ca^{2+} stores was tested by the ability of 10 nM BK to evoke the $K(Ca^{2+})$ channel activity in the absence of external calcium. (*A*) 10 nM BK was added to the Ca^{2+}-free Earle solution containing 2 mM EGTA and zero Ca^{2+}, prior to the addition of 7 μM TSG in the same solution without agonist, as indicated on the top of the trace. (*B*) Same perfusion protocol as in *A*, except that TSG 7 μM was applied prior to BK onto another confluent cell monolayer. Upon cell exposure to BK, the transient activation of $K(Ca^{2+})$ channels appears as fast single-channel openings, superimposed on slow current fluctuations coming from the iK1 channel. (*C*) Inhibitory effect of the maximally effective dose of TSG (20 μM) on the agonist-evoked $K(Ca^{2+})$ channel activation process in Ca^{2+}-free external conditions. Same perfusion protocol as in *B*. Each trace contains 1500 pts of the original current record.

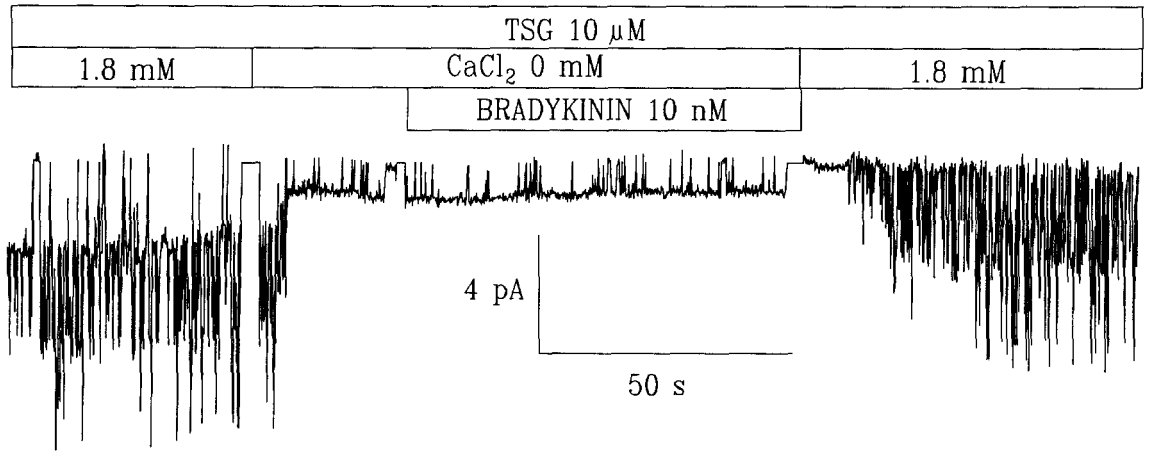

Fig. 4. Absence of the BK-induced single-channel response in conditions where the $K(Ca^{2+})$ channel activity was initiated by exposing BAE cells to 10 μM TSG in the presence of external calcium. Cell-attached current record obtained following a calcium, calcium-free, calcium perfusion protocol (Vp of +30 mV and pipette filled with a high K^+ solution). The initial segment of recording was omitted for clarity; 10 μM TSG was continuously present in the Earle solutions containing either 1.8 mM $CaCl_2$ or 2 mM EGTA and zero Ca^{2+} (bath exchange within 5 sec). External calcium was removed during the $K(Ca^{2+})$ channel activation process induced by TSG, prior to the cell exposure to 10 nM BK. Note the smaller unitary amplitude of the iK1 channel current in the Ca^{2+}-free Earle solution despite the presence of BK. The sustained $K(Ca^{2+})$ channel activity reappeared by superfusing the cells with the normal Earle solution containing TSG. The current trace represents 1500 pts of the original record.

tion that an oscillatory activation process of $K(Ca^{2+})$ channels could be developed in some TSG-treated cells, as described before in some caffeine-treated cells (Thuringer & Sauvé, 1992), may be regarded as evidence for the plausible involvement of a caffeine-sensitive Ca^{2+} store in the response generated by TSG.

Blocking Effects of Procaine

The ability of procaine to block entirely the agonist-evoked Ca^{2+} influx (Thuringer & Sauvé, 1992) was tested on the TSG-induced channel activity to examine whether TSG and BK stimulated the same pathway for the external Ca^{2+} entry. As shown in Fig. 5, the external application of 10 mM procaine reversibly inhibited the sustained activity of $K(Ca^{2+})$ channels recorded in the continuous presence of 10 μM TSG (n = 4). The slow disappearance of channel openings, compared to the drastic inhibition of the BK-evoked channel activity (Thuringer & Sauvé, 1992), occurred with a decrease in the unitary current amplitude and revealed small bursts of channel openings. The removal of procaine from the bath medium was followed by a late reactivation of $K(Ca^{2+})$ channels. Therefore, the action of procaine on the channel activation process elicited by TSG appears similar to that observed on the external Ca^{2+}-dependent phase of the BK-induced channel response.

Discussion

On the basis of the ability of TSG to produce agonist-independent Ca^{2+} influx by acting directly on the $InsP_3$-sensitive Ca^{2+} store, especially in exocrine gland cells (Takemura et al., 1989; Kwan et al., 1990; Foskett et al., 1991), the effects of this Ca^{2+} releasing reagent were examined in order to investigate the relationship between the Ca^{2+} content of endoplasmic cisternae and the Ca^{2+} membrane permeability of endothelial cells.

TSG Induced Ca^{2+} Influx by Emptying Intracellular Ca^{2+} Stores

In agreement with recent data obtained in primary cultures of BAE cells using Fura-2 method (Dolor et al., 1992), our results from cell-attached patch experiments clearly indicate that the cell pretreatment with TSG blocked, in a dose- and time-dependent manner, the ability of BK to cause a transient activation of $K(Ca^{2+})$ channels in the absence of external calcium (*see* Figs. 3*C* and 4). Furthermore, the sustained channel activity evoked by TSG was found to be entirely dependent on the presence of external calcium (*see* Fig. 4). These results suggest that the primary action of TSG is to discharge releasable stores of Ca^{2+} resulting in an activated Ca^{2+} influx, in accordance with a capacitative Ca^{2+} entry model (Putney, 1986; Takemura & Putney, 1989;

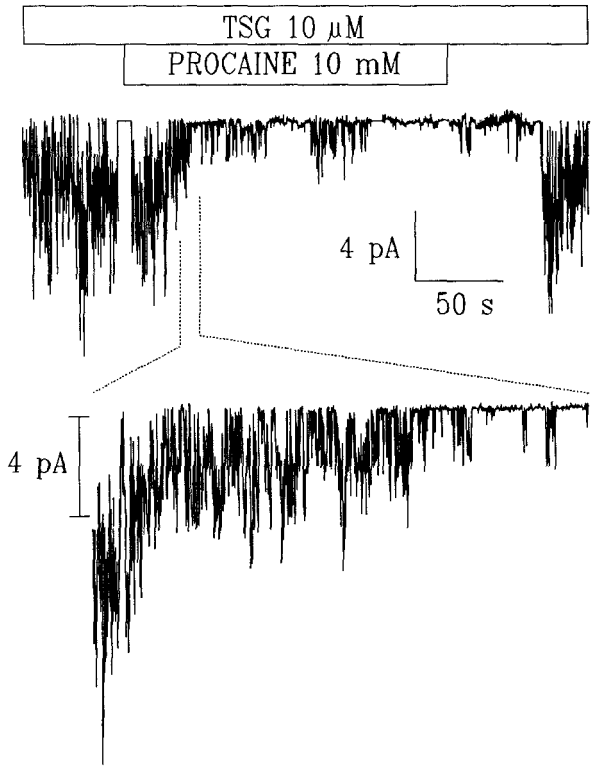

Fig. 5. Blocking effect of procaine on the TSG-induced activation process of the $K(Ca^{2+})$ channels. The addition of 10 mM procaine to the normal Earle solution containing 10 μM TSG, reversibly caused a slow inhibition of the $K(Ca^{2+})$ channel activity recorded in a cell-attached membrane patch (pipette filled with a high K^+ solution and Vp of +30 mV). Some bursts of openings and a reduction in the current jump amplitude of $K(Ca^{2+})$ channels could be observed upon this drug application. Note the slow decrease in amplitude of the unitary currents following the addition of procaine, as shown in the lower trace. The current trace represents 2000 pts of the original record.

Takemura et al., 1989). In this regard, the longer time delay and slower kinetics of the channel activation process evoked by TSG compared to that induced by BK (*see* Fig. 1), may be related to a slower rate of internal Ca^{2+} mobilization induced by TSG as already reported in various cell types (Thastrup, 1990; Dolor et al., 1992). Thus, a rapid depletion of intracellular Ca^{2+} stores does not seem to be required to obtain an external Ca^{2+} influx into BAE cells. In the preceding paper (Thuringer & Sauvé, 1992), we have shown that procaine blocked the external Ca^{2+}-dependent phase of the BK-evoked channel response. Within the framework of the capacitative model (Putney, 1986), this effect of procaine could arise from a partial inhibition of the $InsP_3$-induced Ca^{2+} release (Freay et al., 1989). However, our results indicate that procaine can still block the Ca^{2+}-influx in conditions where the mechanism of Ca^{2+} sequestration into the $InsP_3$-sensitive store was impaired (*see* Fig. 5). In contrast, the caffeine-evoked channel activity (*see* Fig. 9, Thuringer & Sauvé, 1992) appeared poorly affected by this agent. We conclude, therefore, that a part of the effect of procaine can be explained by a direct blockage of the BK- or TSG-stimulated Ca^{2+} entry pathway.

Some differences, however, were observed between the changes in $[Ca^{2+}]i$ detected by Fura-2 (Dolor et al., 1992) and those reflected by the single-channel activity in BAE cells. For instance, no $K(Ca^{2+})$ channel activity was recorded upon TSG application in Ca^{2+}-free external conditions (*see* Fig. 3), whereas smaller TSG doses led to a slow increase in $[Ca^{2+}]i$ to a peak of about 200 nM within 2–2.5 min (Dolor et al., 1992). This difference is likely to arise from the higher Ca^{2+} detection level with $K(Ca^{2+})$ channels (400 nM in excised patches) compared with Fura-2 measurements (*see* Materials and Methods, Thuringer & Sauvé, 1992). Another noticeable difference is the transient action of TSG (7–10 μM) on the $K(Ca^{2+})$ channel activity recorded at room temperature (*see* Fig. 1*A*), whereas a 3-min exposure to lower concentrations (0.2–1 μM) of TSG led to a prolonged elevation of $[Ca^{2+}]i$ (Dolor et al., 1992). Because Fura-2 measurements were performed at 37°C, an effect of temperature on the membrane permeability to TSG cannot be ruled out, and thus, higher doses of TSG may be required at room temperature to promote a rapid and sufficient increase in $[Ca^{2+}]i$ to reach the activation threshold of these channels at the submembrane level.

Possible Implication of a TSG-Insensitive Store to the Cell Variability in Single-Channel Responses Induced by TSG

In the majority of confluent cells tested, micromolar concentrations of TSG produced a sustained activation of $K(Ca^{2+})$ channels which lasted over time periods depending on the dose tested. This channel activation process was occasionally associated with an oscillatory behavior (*see* Fig. 2). Repetitive bursts of channel openings of 60-sec duration separated by silent periods of similar duration (60–70 sec) were generated in some cells exposed to 7–10 μM TSG, whereas a sustained activity was seen systematically at 20 μM TSG. An oscillatory behavior has been recently reported in TSG-treated parotid acinar cells which display external Ca^{2+}-dependent spikes of $[Ca^{2+}]i$ similar to those elicited by agonist (Foskett et al., 1991). Although BAE cells of confluent monolayers rarely displayed an oscillatory channel activity in response to 10 nM BK at room temperature,

single-channel bursts could be induced by external ATP with a frequency dependent on the external concentration of agonist (Sauvé et al., 1988). Such oscillatory behavior has been reported in other endothelial cell preparations which responded in a dose-dependent manner to histamine, thrombin, ATP (Neylon & Irvine, 1990; Jacob, 1991). In particular, Carter and co-workers (1991) have recently reported in pig aortic endothelial cells that $[Ca^{2+}]i$ spikes triggered by external ATP could be reproduced by BK (0.1–5 nM) but with a dose-independent frequency. With the heterogeneity of the agonist-evoked response depending on the cell preparation, numerous hypothetical schemes have been proposed for the mechanism which underlies $[Ca^{2+}]i$ oscillations. For instance, the origin of spiking could be either a periodic inhibition of the Ca^{2+} influx at the membrane level (Laskey et al., 1991) or a repetitive discharge of internal Ca^{2+} stores (Carter, Hallam & Pearson, 1989; Sage, Adams & Van Breemen, 1989; Jacob, 1991). Our results suggest that the oscillation mechanism induced by TSG in BAE cells does not involve directly the $InsP_3$-sensitive store, but could take place in another Ca^{2+} store possessing a TSG-insensitive Ca^{2+}-uptake mechanism as previously suggested in parotid acinar cells (Foskett et al., 1991; Foskett & Wong, 1991). One likely possibility for endothelial cells could be the caffeine-sensitive store that we have identified previously (Thuringer & Sauvé, 1992).

On the basis of the present results, we conclude that the TSG-sensitive Ca^{2+} stores include the $InsP_3$-sensitive store, the emptying of which might cause a sustained Ca^{2+} entry from the extracellular medium, as described in Putney's capacitative model (1986). Because a significant amount of Ca^{2+} has been shown to be mobilized by TSG in endothelial cells pretreated with BK (Dolor et al., 1992), we cannot exclude the possible existence of an $InsP_3$-insensitive pool which would constitute a fraction of the pool sensitive to TSG, as reported in rat hepatocytes (Thastrup et al., 1990). In accordance with the results obtained in parotid acinar cells (Foskett et al., 1991; Foskett & Wong, 1991), the oscillatory behavior observed in some BAE cells in response to TSG involves a Ca^{2+}-induced Ca^{2+}-release mechanism from an $InsP_3$- and TSG-insensitive Ca^{2+} store.

The actions of reagents used in our studies are summarized in a simplified scheme shown in Fig. 6, where the intracellular Ca^{2+} movements are restricted between three subcompartments only: the cytosol, $InsP_3$-sensitive and caffeine-sensitive stores. These intracellular Ca^{2+} stores are separated in order to explain the transient single-channel response induced by caffeine alone and the subsequent release of Ca^{2+} following the additional application

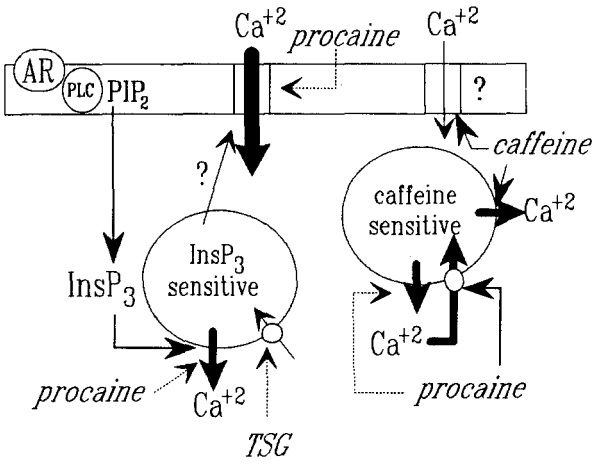

Fig. 6. Hypothetical model for Ca^{2+} signaling transduction in endothelial cells. The $InsP_3$-sensitive and caffeine-sensitive Ca^{2+} stores are presumed to be distinct. The cell stimulation by BK stimulates the chemical cascade involving a G-protein activation of phospholipase C-mediated phosphatidyl inositol 4,5-biphosphate (PIP_2) hydrolysis, yielding rapidly $InsP_3$ and diacylglycerol (DAG). The transient production of $InsP_3$ is correlated with Ca^{2+} release from the $InsP_3$-sensitive store of which the rapid depletion causes an increased Ca^{2+} influx into the cytosol. The rapid rise of $[Ca^{2+}]i$ triggers a release of Ca^{2+} from the secondary store by Ca^{2+}-induced Ca^{2+}-release probably underlying $[Ca^{2+}]i$ oscillations. TSG depletes the $InsP_3$-sensitive Ca^{2+} store by specific inhibition of the microsomal Ca^{2+}-ATPase activity, and consequently causes a sustained external Ca^{2+} entry. Procaine inhibits patially the $InsP_3$-induced Ca^{2+} release and blocks directly the Ca^{2+} entry pathway activated by the emptying of the $InsP_3$- and TSG-sensitive Ca^{2+} store. In contrast, procaine exerts an inhibitory effect on the caffeine-induced Ca^{2+} release depending on the Ca^{2+} content of the store, but would not affect the Ca^{2+} influx activated by caffeine.

of BK in Ca^{2+}-free external conditions (Thuringer & Sauvé, 1992). TSG would act exclusively on the $InsP_3$-sensitive store by favoring a Ca^{2+} leak pathway perhaps through an inhibitory effect on the Ca^{2+} reuptake process into this store. The ability of some cells to produce oscillatory single-channel responses to TSG would be explained by the concomitant activation of a Ca^{2+}-induced Ca^{2+}-release process from the caffeine-sensitive store. This model requires a direct control of the external Ca^{2+} influx by the fullness of the $InsP_3$-sensitive Ca^{2+} store to account for the external Ca^{2+} dependency of the channel activation process induced by TSG, in agreement with the previous findings reported by Dolor and coworkers (1992). Although the Ca^{2+} entry pathway and the nature of its control remain unknown, the TSG effects do not support an important contribution to the stimulated Ca^{2+} influx coming from stretch-activated channels (Lansman, Hallam & Rink, 1987) or receptor-operated channels (Johns et al., 1987) which could be coupled to second messen-

gers such as Ins (1,3,4,5) P_4 (Lückhoff & Clapham, 1992). Three distinct sites of action of procaine must be considered. In accordance with previous observations of Freay and co-workers (1989), procaine partially inhibits the $InsP_3$-induced Ca^{2+} release, perhaps through a direct effect on the Ca^{2+} release channel. Its ability to antagonize the caffeine-induced Ca^{2+} release would be dependent on the amount of Ca^{2+} in the caffeine-sensitive store in such a way that an increase in Ca^{2+} content into this store reduces the blocking effect of procaine. Because procaine blocked the external Ca^{2+}-dependent phase of the BK-evoked channel activity as well as that induced by TSG, procaine can also exert a direct blockage of the Ca^{2+} entry stimulated by BK and TSG. Although the exact action of procaine has to be clarified, the lack of its inhibiting effect on the sustained phase of the caffeine-evoked Ca^{2+} response allows us to suppose that caffeine leads to the activation of an external Ca^{2+} entry pathway which is poorly sensitive to procaine compared to that mainly triggered by the agonist. Further experiments are in progress to confirm this model.

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