Progression from Homologous to Heterologous Desensitization of Contraction in Gastric Smooth Muscle Cells

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ABSTRACT

Acute desensitization of contraction and its relative mechanisms have been studied in smooth muscle cells isolated from guinea pig stomach. Desensitization was induced by pre-exposure of the cells to one of the excitatory neuropeptides linked to the phospholipase C intracellular cascade, i.e., cholecystokinin (CCK), gastrin-releasing peptide, and Substance P. Desensitization was homologous after a 30-s pre-exposure and heterologous if pre-exposure lasted for 5 min or longer. Homologous desensitization was studied in a more detailed way after pre-exposure to CCK. Preincubation with increasing concentrations of CCK (10 pM–1 nM) induced a progressive rightward shift of the dose-response curves associated with both a decrease in potency (ED50 4.5 pM–2.2 nM) and a maximum response that were not related to a modification of response kinetics. After brief pre-exposure to 1 nM CCK (Dmax), an inhibition of contraction was observed in response to an identical dose of CCK (45.1 ± 8.6%) in. The decreased response being associated with an inhibition of inositol phosphates and [Ca2+]i mobilization. Both inositol trisphosphate (InsP3)-induced contraction and [Ca2+]i mobilization were inhibited to a lesser extent than CCK-induced responses. Any longer pre-exposure of cells to one of the above-mentioned neuropeptides caused heterologous desensitization, with an observed inhibition of contraction in response to all tested agonists (CCK, 60.3 ± 5.9%; gastrin-releasing peptide: 56.7 ± 3.5%; Substance P, 60.6 ± 6.5%). A similar decrease was observed in InsP3-induced contractions resulting in a desensitization of the InsP3 response as well. Full recovery of contractile responses appeared within 30 min from the end of preincubation, thus indicating that degradation of membrane receptors did not occur. Although pre-exposure of the cells to protein kinase C inhibitor GF109203X did not modify CCK-induced homologous desensitization, it blocked CCK-induced heterologous desensitization. This study demonstrates that excitatory phospholipase C-coupled enteric neuropeptides induce a time-dependent homologous as well as heterologous desensitization of smooth muscle contraction occurring at receptor and postreceptor levels.

The interaction of agonists with plasma membrane receptors triggers the activation of an intracellular cascade of biochemical events that leads to the final biological response. A single cell that possesses multiple membrane receptors for such agents (Makhlouf, 1987; Gardner and Jensen, 1993) could be exposed simultaneously to several of them. Inside the cell are various feedback mechanisms that integrate and regulate overall receptor activation. The most widespread regulatory mechanism is desensitization, which consists of a loss of responsiveness to an agonist that develops after prior or persistent exposure to the same, or to another, agonist. Desensitization is involved in the regulation of cellular responses to different agonists and in the prevention of cellular overstimulation, thus protecting the cells or tissues from abnormal stimuli. All biological systems in which desensitization occurs exhibit membrane receptors that mediate the effects induced by a specific agonist (Sibley and Leftkowitz, 1985).

Depending on its time of appearance, desensitization can be acute or chronic. Furthermore, according to the agonist that triggers the phenomenon, it can be either homologous or heterologous. These classifications are complementary. Acute desensitization appears rapidly (seconds/minutes) and disappears quickly after the removal of the stimuli, which seems to be due to an uncoupling between the receptors and the intracellular cascade with a consequent loss of membrane receptor function. Chronic desensitization is observed after hours or days of exposure to the agonist and intracellular proteolytic degradation of the receptors occurs, requiring a

ABBREVIATIONS: CCK, cholecystokinin; DAG, 1,2-diacylglycerol; Fura-2-AM, 1-[2-carboxyoxazol-2-yl]-6-aminobenzofuran-5-oxyl-2-(2'-amino-5'-methylphenoxyl)-ethane-N,N,N'-tetraacetic acid, sodium salt; GF109203X, 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl) maleimide; GRP, gastrin-releasing peptide; InsPs, inositol phosphate(s); InsP3, inositol trisphosphate; PKC, protein kinase C; PLC, phospholipase C; SP, substance P.
resynthesis to restore normal cellular response. Desensitiza-
tion can also be classified as agonist-specific (homologous) and
agonist-nonspecific (heterologous), the former being re-
stricted to the ligand used as a desensitizer and stimulus. In
heterologous desensitization cells become refractory to sev-
eral classes of agonists (Lohse, 1993).

As for acute desensitization, the most extensively studied
receptor is the beta-2 adrenoceptor (Hausdorff et al., 1990),
a G-coupled receptor that stimulates cellular responses by
activating adenylate cyclase with a subsequent increase in
intracellular cAMP. Much less is known regarding the desen-
sitization of G protein-coupled receptors that stimulate cel-
lar responses by activating the phospholipase C (PLC)-
dependent intracellular pathway. Desensitization of this
class of G protein receptors appears to be a multistep phe-
nomenon (Chuang et al., 1996). There is considerable varia-
tion in the susceptibility of this class of receptors to desensi-
tization as well as in the intracellular mechanisms involved
in the phenomenon (Wojcikiewicz et al., 1993; Logston, 1994).
It appears from recent studies that these receptors can be
acutely desensitized by multiple mechanisms, causing either
homologous or heterologous desensitization, and that differ-
ent desensitization mechanisms are used by a given receptor
in different cells (Hishinuma and Uchida, 1989; Battistini et
al., 1990; Bolger et al. 1992; Lods et al., 1995).

A group of agonists that interacts with G protein-coupled
receptors linked to phosphatidylinositol hydrolysis and to
Ca\(^{2+}\)-dependent intracellular mechanisms is represented by
the peptidergic excitatory neurotransmitters present in the
enteric nervous system. These neurotransmitters, some of
which are coexpressed by the same neurons, may activate
smooth muscle cells, causing contraction, by a combined ac-
tion (plurichemical transmission) (Furness et al., 1989; Tor-
soli and Severi, 1993). After neural stimulation, various neu-
rotransmitters may be released by enteric nerves, and, for
this reason, smooth muscle cells can be exposed simulta-
neously to multiple agonists. Several mechanisms of regula-
tion then must occur in these cells to prevent overstimula-
tion, but few studies have been carried out in this area.
Muscarnic receptors have been the most extensively studied
(Mita and Uchida, 1987; Hishinuma et al., 1990).

The aim of the present study was to investigate acute
desensitization of contraction caused by excitatory enteric
neuropeptides in smooth muscle cells isolated from guinea
pig stomach. The mechanisms involved in receptor desensi-
tization were studied by evaluating contractions and intra-
cellular inositol phosphate (InsP) and Ca\(^{2+}\) levels, together
with selective activation of the different steps in the intra-
cellular cascade. Our data show that smooth muscle cells
are desensitized by enteric excitatory neurotransmitters and,
depending on the duration of pre-exposure of smooth muscle
cells to an agonist, that desensitization was either homolo-
gous or heterologous and occurred at receptor and postrecep-
tor levels. The dual pattern of desensitization was associated
with a different relative involvement of the various intracel-
lar mechanisms responsible for the contractile response.

Materials and Methods

Materials

Chemicals. Cholecystokinin (CCK), gastrin-releasing peptide
(GRP), and Substance P (SP) were obtained from Bachem (Bubendorf,
Switzerland); Fura-2-free acid, 1-(2-carboxyoxazol-2-yl)-6-aminobenzo-
furan-5-oxyl]-2-(2’-aminophenyl)ethane-N,N,N’,N’-tetraacetic acid, sodium salt
(Fura-2-AM), intrinol trisphosphate (InsP\(_3\)), 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-
3-yl)maleimide (GF109203X), and saponin were purchased from Calbiochem (La Jolla, CA); collagenase CLS Type II and soybean
trypsin inhibitor were obtained from Worthington (Freehold, NJ);
Eagle’s minimum essential amino acid mixture was obtained from Gibco (Paisley, UK); d-\(^{3}H\)-inositol (specific activity, 814
GBq/mmol) was obtained from DuPont-NEN (Bad Hamburg,
Germany); and phorbol 12-O-tetradecanoylphorbol-13-acetate,
HEPES, Dowex 1X-200 (100–200 mesh), and all other reagents
were obtained from Sigma (St. Louis, MO).

Animals. Male guinea pigs (200–300 g, body weight) were pur-
cashed from Charles River Italy (Milan, Italy).

Methods

Preparation of Smooth Muscle Cells. Smooth muscle cells
were isolated from guinea pig stomach as described previously (Bitar
and Makhlouf, 1992). In brief, muscle strips, free of mucosa, were
incubated at 31°C for two successive 45-min periods in 15 ml of
HEPES medium, pH 7.4, containing 0.1% collagenase (150 U/ml).
The composition of the medium was as follows: 115 mM NaCl, 5.8
mM KCl, 2.1 mM KH\(_2\)PO\(_4\), 2.9 mM CaCl\(_2\), 0.6 mM MgCl\(_2\), 25 mM
HEPES, and 14 mM glucose. The medium also contained 0.01%
soybean trypsin inhibitor and 2.1% essential amino acid mixture.
At the end of the second incubation period, the muscle strips were
washed with enzyme-free medium on 500-μm Nitex mesh and resus-
pended in the same medium for 30 min to allow spontaneous disper-
sion of cells. In some experiments, cell dispersion was accelerated by
gentle suction of the suspension using an inverted 10-ml pipette. The
cells then were harvested by filtration through 500-μm Nitex mesh
and then centrifuged for 5 min at 350g to adjust the selected cellular
concentration required for desensitization protocols (i.e., 10\(^{4}\) cells/ml in
contraction experiments or 10\(^{6}\) cells/ml in InsP and Ca\(^{2+}\) mea-
surements).

Only cells spontaneously dispersed in enzyme-free medium were
used for biological measurements and desensitization protocols. The
concentration of cells was measured using a standard hemocytome-
ter, and cell viability was checked by trypan blue exclusion. For
experiments, only preparations with 95% unstained cells were used.

When permeabilization was required, dispersed smooth muscle cells
were centrifuged at 350g for 5 min and resuspended in a medium
with a cytosolic-like composition especially in terms of Ca\(^{2+}\) ions
(180 nM), namely: 20 mM NaCl, 100 mM KCl, 5 mM MgSO\(_4\), 20 mM
HEPES, 0.5 mM CaCl\(_2\), 1 mM EGTA, and 1% bovine serum albumin,
pH 7.2 (Bitar et al. 1986). Cell permeabilization was achieved by
incubation with saponin (35 μg/ml) for 5 min. The cells were then
centrifuged at 350g for 5 min, washed free of saponin, and resus-
pended in the cytosolic-like medium, to which ATP (1.5 mM) and
antimycin (10 μM) had been added together with an ATP-regener-
ating system, consisting of creatine phosphate (5 mM) and creatine
phosphokinase (10 U/ml).

Measurement of Contraction. Contraction was measured in
suspensions of smooth muscle cells as described previously (Bitar
and Makhlouf, 1982). Cell suspension (0.5 ml) was added to 0.2 ml of
incubation medium containing the agent to be tested, and the reac-
tion was stopped at fixed time intervals by adding acrolein (final
concentration, 1%). The interval was timed to elicit a peak con-
traction with each agent tested (30 s with receptor agonists; 5 s with
InsP\(_3\)). For measurement of control cell length, the agent was omit-
ted and an equivalent volume of medium was added. In control
samples and upon addition of test agents, the length of 50 cells in
sequential microscopic fields was measured by image-scanning mi-
croscopy (Lasico; Los Angeles, CA). Contraction was expressed as
the mean percentage decrease in cell length with control length
taken as 100%.
Measurements of Cytosolic-Free Ca\(^{2+}\) (\([\text{Ca}^{2+}]_i\)) and Inositol Trisphosphate (InsP\(_3\)) were determined in intact and permeabilized smooth muscle cells, as described previously (Roux et al., 1992; Murthy et al., 1993), using the fluorescent Ca\(^{2+}\) dyes Fura-2-AM and Fura-2-free acid, respectively. For \([\text{Ca}^{2+}]_i\) measurements, dispersed intact cells were resuspended in HEPES medium consisting of: 125 mM NaCl, 5 mM KCl, 10 mM HEPES, 0.5 mM MgSO\(_4\), 1 mM CaCl\(_2\), 5 mM glucose, 20 mM taurocholate, 5 mM sodium pyruvate, and 5 mM creatine, pH 7.4. Two milliliters of cell suspension (10\(^6\) cells/ml) was incubated with Fura-2-AM (2 \(\mu\)M) for 30 min at 31°C, centrifuged at 350g for 20 min, and resuspended in 2 ml of HEPES medium. Fluorescence was measured within 5 min. Maximal fluorescence was determined after addition of 100 ng/ml digitonin (81 \(\mu\)M) and minimal fluorescence was determined after addition of 1.25 mM EGTA plus 60 mM Tris.

\([\text{Ca}^{2+}]_i\) was also measured in permeabilized cells. For these experiments, dispersed muscle cells were resuspended in a cytosolic-like medium consisting of: 20 mM NaCl, 100 mM KCl, 5 mM MgSO\(_4\), 20 mM HEPES, 0.5 mM CaCl\(_2\), and 0.05 mM EGTA, pH 7.2. Cells were then permeabilized by incubation with saponin 35 \(\mu\)g/ml for 5 min at 31°C, washed free of saponin, and then resuspended in the cytosolic-like medium to which ATP (3 nM), phosphocreatine (10 mM), creatine phosphokinase (10 \(\mu\)M), and antimycin (10 \(\mu\)M) had been added. Fura-2-free acid (1 \(\mu\)M) was then added to 2 ml of permeabilized cells (10\(^6\) cells/ml) contained in the quartz cuvette, and fluorescence was monitored as for intact cells. Maximal fluorescence was determined after addition of 1 mM Ca\(^{2+}\), and minimal fluorescence was determined after addition of 4 mM EGTA. Although indicated as \([\text{Ca}^{2+}]_i\), the Ca\(^{2+}\) levels reflected concentrations in the extracellular medium and the cytosol of permeabilized muscle cells.

Fluorescence was monitored at an emission wavelength of 510 nm using a Perkin-Elmer LS3 fluorescence spectrofluorimeter (Norwalk, CT) with excitation wavelength alternating between 340 and 380 nm, using a dissociation constant of 224 nM, as described by Bitar and Makhlouf (1982). CCK-induced desensitization therefore was evaluated during the second incubation. Statistical Analysis. Results were expressed as mean \pm S.E. of \(n\) experiments, and the data were calculated with Student’s \(t\) test (\(p < 0.05\) was considered statistically significant). Cells for each experiment were obtained from different animals. The agonist concentration causing half-maximal response, indicated as ED\(_{50}\), was obtained by linear regression analysis.

**Results**

Smooth muscle cells isolated from guinea pig stomach had a mean cellular length of 101.0 \(\pm\) 2.2 \(\mu\)m and a maximal contraction of 24.9 \(\pm\) 1.5% in response to 1 nM CCK.

Control experiments were performed initially to verify whether preincubation of smooth muscle cells, followed by the two wash-out steps, could cause modifications of the basal experimental conditions. For this purpose, resting cell length before and after preincubation with or without a given peptide was measured. In the absence of the agonist, only a small reduction of cell length (3.0 \(\pm\) 1.1%) from the initial value was observed after preincubation and subsequent washings. Agonist preincubation also did not induce any significant modification of cell length. In fact, no difference was observed between resting cell length of cells preincubated with the peptide after removal of the agonist (95.3 \(\pm\) 3.0 \(\mu\)m) and cells preincubated without the agonist (98.3 \(\pm\) 3.0 \(\mu\)m), showing that no residual contraction was present after agonist preincubation and the washings that followed.

When smooth muscle cells were desensitized with increasing doses of CCK (10 \(\mu\)M–1 \(\mu\)M), a progressive shift to the right of the dose-response curves to CCK was observed in these cells. As shown in Fig. 1, a decrease in both maximal response and in potency of the peptide was observed in cells preincubated for 15 min with submaximal (10 \(\mu\)M; ED\(_{50}\); 4.5 \(\mu\)M) or maximal dose of CCK (1 nM; ED\(_{50}\); 9 \(\mu\)M). In cells preincubated with supramaximal doses of CCK (10 nM–1 \(\mu\)M) there was a further decrease in potency of the peptide (ED\(_{50}\); from 45 \(\mu\)M to 2.2 \(\mu\)M) associated with a rightward shift in the maximal dose. In control cells, namely, in cells preincubated in the absence of any peptide, CCK had a potency (ED\(_{50}\); 1.2 \(\mu\)M) similar to that normally observed in isolated guinea pig gastric smooth muscle cells (Bitar and Makhlouf, 1982). CCK-induced desensitization therefore was strongly dose-dependent.

To investigate whether this decreased response of desensitized cells might be due to a difference in time required to reach peak contraction, response kinetics were studied in both control cells (no addition) and cells preincubated with 1 nM CCK (Fig. 2). No differences were observed in the time course of response. In both preparations, peak contraction...
was observed at 30 s and was followed by a decrease of the response to approximately 40% of maximal contraction, which remained constant for up to 2 min.

The time course of desensitization then was evaluated. Smooth muscle cells preincubated with 1 nM CCK for up to 15 min displayed a progressive time-dependent decrease in subsequent CCK-induced maximal contraction (Fig. 3). The decrease in contraction was detectable after 30 s of preincubation, increased during the following 5 min, and then remained constant. In cells preincubated without agonist, no inhibition of maximal contraction was observed.

To elucidate whether CCK caused homologous or heterologous desensitization, smooth muscle cells were preincubated with either different doses of CCK or a maximal dose of CCK for different times to be re-exposed subsequently to CCK, GRP, or SP. A short, 30-s pre-exposure to 1 nM CCK caused only a decrease of subsequent response to CCK without affecting those maximal contractile responses caused by the other peptides, thus indicating homologous desensitization (Table 1). A longer pre-exposure (5 min) inhibited instead to the same extent CCK-, GRP-, and SP-induced contractions (Table 1), thus indicating heterologous desensitization. A similar trend was observed with submaximal doses of CCK (data not shown). To exclude that a heterologous desensitization could have been missed after short CCK pre-exposure by the use of maximal agonists concentrations, GRP and SP dose-response curves were constructed before and after a 30-s preincubation with 1 nM CCK. Similar potency for either peptide was observed before (ED50: GRP = 10 pM and SP = 20 pM) or after short-term desensitization to CCK (ED50: GRP = 11 pM and SP = 35 pM), thus confirming that the homologous pattern occurred on maximal responses. The observed time dependence of CCK-induced desensitization then was associated with different patterns of desensitization.

To elucidate the cellular mechanisms responsible for this dual pattern of desensitization, the different intracellular steps involved in contractile responses were investigated using direct activators and inhibitors of intracellular responses and measuring intracellular mediators. For this purpose, InsP accumulation and Ca++ mobilization induced by CCK were evaluated in control and desensitized cells. In control cells preincubated without agonist, CCK induced an intracellular increase of [Ca++] five times greater than basal values, an increase similar to that observed in intact gastric smooth muscle cells (5.2 ± 1.5 times). InsP3 induced an increase of...
intracellular [Ca\(^{++}\)] similar to that observed in response to CCK. In permeabilized cells pre-exposed for 30 s to 1 nM CCK, a decrease in intracellular [Ca\(^{++}\)] mobilization was observed in response to CCK and InsP\(_3\). The decrease in InsP\(_3\)-induced mobilization was significantly lower than the decrease in CCK-induced response. On the contrary, in permeabilized cells pre-exposed for 5 min to the same dose of CCK, CCK- and InsP\(_3\)-induced responses were almost equally inhibited. In desensitized cells the inhibition of CCK-induced intracellular [Ca\(^{++}\)] mobilization was significantly lower than the decrease in CCK-induced response. On the contrary, in permeabilized cells pre-exposed for 5 min to the same dose of CCK, CCK- and InsP\(_3\)-induced responses were almost equally inhibited. In desensitized cells the inhibition of CCK-induced intracellular [Ca\(^{++}\)] mobilization was significantly lower than the decrease in CCK-induced response.

To further investigate the time dependence observed in InsP\(_3\)-induced Ca\(^{++}\) release in desensitized cells, the effect of exogenous InsP\(_3\) on smooth muscle cell contraction in control and desensitized cells was evaluated. For this purpose, isolated smooth muscle cells were previously permeabilized, then desensitized for 30 s or 5 min with 1 nM CCK, and, after two washings to remove the agonist, re-exposed to 1 nM CCK or to 1 \(\mu\)M InsP\(_3\). Control experiments were carried out to evaluate the effect of cell permeabilization on the contractile response and on the time dependence of desensitization. In control cells (no addition), CCK-induced contraction in permeabilized cells (24.7 ± 1.4%) was similar to the response observed in intact cells (22.5 ± 1.3%). Furthermore, permeabilized cells pre-exposed for different times to a maximal dose of CCK showed a time-dependent decrease in contraction (14.6 ± 3.9% after 30 s; 9.0 ± 2.0% after 5 min) similar to that observed in intact desensitized cells (13.8 ± 1.8% after 30 s; 9.4 ± 0.9% after 5 min). Smooth muscle cell membrane permeabilization therefore did not modify the time dependence of CCK-induced desensitization. Moreover, in permeabilized cells, the time dependence of CCK-induced desensitization was associated with different patterns of desensitization. A 30-s pre-exposure to CCK did not modify GRP- nor SP-induced contraction (20.3 ± 2.4% and 25.9 ± 1.7%, respectively), while longer pre-exposure inhibited both these responses (13.5 ± 2.4% and 13.6 ± 1.4%, respectively). In permeabilized cells, contraction induced by maximal doses of CCK and InsP\(_3\) then was evaluated (Fig. 5). The two agonists, which activate contraction at different sequential steps, caused a similar maximal response in control permeabilized cells, but this response was inhibited in desensitized cells. In desensitized cells, the pattern of response to the two
agonists depended on the time of pre-exposure to CCK. Comparing the contraction induced by the two agonists in permeabilized cells pre-exposed for 30 s to 1 nM CCK, the response induced by InsP$_3$ was reduced to a lesser extent than the contraction induced by CCK. A 5-min preincubation with 1 nM CCK instead caused an equal reduction in CCK- and InsP$_3$-induced contractions.

To investigate the role played by PKC in desensitization, smooth muscle desensitization experiments were carried out before or after a 60-min preincubation with 10 μM GF109203X, a PKC inhibitor (Toullec et al., 1991). For this purpose CCK- and SP-induced maximal contractions were evaluated in desensitized cells that had been preincubated with or without the PKC inhibitor. This pre-exposure to GF109203X was effective in inhibiting PKC in gastric smooth muscle cells because the contraction induced by 1 nM PKC-stimulating phorbol ester phorbol 12-O-tetradecanoylphorbol-13-acetate (14.1 ± 1.1%) was inhibited when the cells were preincubated with GF109203X (8.6 ± 0.7%).

Pretreatment of smooth muscle cells with the PKC inhibitor did not modify CCK-induced homologous desensitization as no differences were observed in the selective inhibition of CCK-induced contraction in smooth muscle cells pre-exposed for 30 s or 5 min to 1 nM CCK (Fig. 6). The time dependence of CCK-induced homologous desensitization was not PKC-dependent. On the contrary, CCK-induced heterologous desensitization was PKC-dependent. In fact, the inhibition of the SP-induced maximal contraction observed after 5-min pre-exposure to CCK in cells not treated with the specific PKC inhibitor was overcome in 5-min desensitized cells pretreated with GF109203X (Fig. 6). The effect of GF109203X pretreatment on InsP$_3$-induced contraction was evaluated in normal

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**Fig. 4.** Basal (■) and stimulated [Ca$^{2+}$]$_i$ levels in response to 1 nM CCK (□) and 1 μM InsP$_3$ (●) in permeabilized cells previously pre-exposed to no agonist or to CCK for 30 s or 5 min (data are the mean ± S.E. of 10–11 experiments; *p < .05 versus CCK-induced release for each treatment).

**Fig. 5.** CCK, 1 nM (■), and InsP$_3$, 1 μM (●), induced contraction of permeabilized guinea pig gastric smooth muscle cells preincubated with no addition or with 1 nM CCK for 30 s or 5 min (data are the mean ± S.E. of three to five experiments; *p < .05 versus CCK-induced contraction for each treatment).
and desensitized cells. For this purpose, smooth muscle cells were pre-incubated for 60 min with or without 10 μM GF109203X, then permeabilized and finally incubated for 5 min in the absence or presence of 1 nM CCK. Pretreatment with the PKC inhibitor reduced the inhibition of InsP₃-induced contraction observed after a 5-min pre-exposure to CCK (Table 3). These data parallel the previous observation on restoration of SP-induced contraction in desensitized cells pretreated with GF109203X and desensitized by 5-min pre-incubation with 1 nM CCK.

Finally, to examine whether CCK-induced desensitization of contraction was a reversible phenomenon, smooth muscle cells were pre-exposed for 15 min to 1 nM CCK and, from the end of the second washing, CCK-induced contraction was measured until restoration of the maximal response at 15-min intervals. As shown in Fig. 7, 15 min after the end of the washings, contraction in response to a maximal dose of CCK was already higher than that observed in the same cells at

<table>
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<th>TABLE 3</th>
<th>InsP₃-induced contraction in permeabilized smooth muscle cells, pretreated with or without PKC inhibitor GF109203X²</th>
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<tr>
<td>No Addition</td>
<td>CCK, 1 nM</td>
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<tr>
<td>− PKC inhibitor</td>
<td>23.8 ± 2.5</td>
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<tr>
<td>+ PKC inhibitor</td>
<td>21.4 ± 0.2</td>
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Cells were pretreated for 60 min with or without 10 μM GF109203X and then preincubated for 5 min in the absence or presence of 1 nM CCK.

² Data are expressed as percent decrease ± S.E. in cell length (n = 3–5).
time 0 (i.e., at the end of preincubation). After 30 min, a complete recovery of contractile response in desensitized smooth muscle cells was detected. Thirty minutes after the first incubation, maximal CCK-induced contraction in previously desensitized cells was similar to that observed in cells preincubated without agonist.

**Discussion**

In this study we have shown that pre-exposure of smooth muscle cells to CCK, GRP, and SP, whose receptors couple to phosphatidylinositol hydrolysis and to intracellular Ca\(^{2+}\)-dependent mechanisms, caused desensitization of contractile responses that, depending on the time of preincubation, was either homologous or heterologous. A 30-s preincubation with CCK caused an inhibition of subsequent responses to CCK only, without affecting GRP- and SP-induced responses. A 5-min or longer pre-exposure to CCK inhibited GRP- and SP-induced contraction as well, resulting in a heterologous pattern of desensitization. The progression from homologous to heterologous desensitization was not related to the dose of agonist present in the preincubation medium. A similar behavior has been observed in endothelial (Wilkinson et al., 1994) but not in other cells (Rahman and Neuman, 1993; Sjödin and Gylfe, 1994). Thus, in gastric smooth muscle cells, the interaction of CCK with its specific receptor first activates contraction and almost simultaneously inactivates the response to CCK itself. Subsequently, desensitization to other agonists acting through the same intracellular pathway occurs. Such a rapid onset of homologous desensitization may be a characteristic of smooth muscle tissue because a similar latency of appearance has been observed in guinea pig taenia coli (Hishinuma et al., 1993). Desensitization of contractile response also occurs when cells are exposed continuously to the same peptide. In fact, after reaching peak contraction a decrease in response was observed in kinetic studies that were performed in the continuous presence of the agonist.

In smooth muscle cells, homologous and heterologous desensitization occurs at receptor and postreceptor levels. Excitatory enteric neuropeptides induce smooth muscle contraction by interacting with specific membrane receptors, which, in turn, activate PLC via a regulatory G protein. This enzyme is responsible for hydrolysis of the plasma-membrane phospholipid phosphatidylinositol 4,5-biphosphate, thus generating the second messengers Ins\(_P_3\) and 1,2-diacylglycerol (DAG). Ins\(_P_3\) triggers the release of Ca\(^{2+}\) from intracellular stores producing a rise in [Ca\(^{2+}\)]\(_i\), whereas DAG activates cytosolic PKC. An additional aim of this study was to clarify the relative role of different intracellular mechanisms involved in the observed dual pattern of desensitization.

Homologous desensitization, which was studied in more detail after pre-exposure to CCK, appeared after a pre-exposure of 30 s and was reversible and dose-dependent. Both membrane receptors and intracellular mechanisms were involved in this pattern. The decrease in potency and efficacy observed in CCK dose-response curves in desensitized cells suggests an involvement of membrane receptors in the inhibition of contractile responses. Desensitization caused by increasing concentrations of CCK caused variations in dose-response curves that were anomalous with respect to predictions of classical receptor theory (Kenakin, 1985). First, there was a decrease both in potency and in efficacy of the contractile agent, while more intense desensitization was limited to a further rightward shift of the curves without a further reduction in the maximal amplitude of the response. It should be noted from previous work (Makhlouf, 1987) and as shown in Fig. 1 that dose-response curves of contractile agents in smooth muscle cells are biphasic, with the maximal contractile response being strongly affected by inhibition caused by supramaximal stimuli. Upon removal of the ago-
nist, there was a rapid recovery of the contractile response, indicating that receptor modification was temporary. Because the response recovered fully within 30 min from the end of preincubation, degradation of membrane receptors probably can be excluded. It has been postulated that homologous desensitization, as opposed to heterologous desensitization, is a result of receptor modifications not involving alterations of intracellular mechanisms triggered by receptor activation (Triggle, 1980). The broad range of CCK concentrations (10 pM–1 μM) that induce desensitization of smooth muscle cell response, however, might reflect an involvement of more than one mechanism, as observed for bombesin on pancreatic acinar cells (Sjödin and Gyölfé, 1994). In fact, in our cell system, intracellular mechanisms modulate homologous desensitization. One mechanism might be a decrease in InsPs formation, because the InsPs increase in response to CCK was inhibited after pre-exposure of the cells for 30 s to 1 nM CCK. Reduced PLC-catalyzed hydrolysis with a subsequent decrease in InsP3 and DAG production has been observed in other cellular systems (Larsson and Simonsson, 1993; Shinohara and Kawasaki, 1994; Servant et al., 1995). The reduced InsPs production is reinforced further by the observation that in smooth muscle cells pre-exposed to CCK for 30 s, the InsP3-induced contractile response as well as intracellular Ca2+ mobilization were inhibited to a lesser extent than were CCK-induced responses. The higher degree of inhibition observed in InsP3-induced Ca2+ mobilization than in InsP3-induced contraction might be due to desensitization of the former with respect to the latter (Masters et al., 1985). The inhibition observed in InsP3-induced responses, however, suggests that some additional intracellular mechanism is involved in homologous desensitization of smooth muscle cells, acting most likely at the level of InsP3 metabolism and/or of InsP3 receptor desensitization (Ferris et al., 1992; Alblas et al., 1995). PKC, instead, seems not to play an active role in the induction of acute homologous desensitization in gastric smooth muscle cells because the PKC inhibitor GF109203X did not alter the pattern of desensitization of the contractile agent used as desensitizer (i.e., CCK). A similar result has been obtained in other cell types (Walsh et al., 1993), although it is well known that specific agonist-induced desensitization can differ in the various cellular systems (Shih and Malbon, 1994; Lods et al., 1995) and that agonists can activate different regulatory mechanisms in the same cells (Kurose and Lefkowitz, 1994).

A longer pre-exposure of the cells to the agonist caused an increase in the extent of homologous desensitization and the appearance of heterologous desensitization. In fact, after a 5-min pre-exposure to CCK, inhibition of subsequent CCK-induced contraction was higher than that observed after a 30-s preincubation, and GRP- and SP-induced contractions were inhibited as well. Other regulatory mechanisms appeared to be involved in the inhibition of responses caused by a longer pre-exposure of smooth muscle cells to CCK. Most of the effects observed, after a 5-min pre-exposure, appeared to be PKC-dependent. Preincubation with the PKC inhibitor GF109203X, while failing to remove homologous inhibition to CCK, abolished heterologous desensitization to SP and reversed the inhibition of InsP3-induced response. Inhibition of PKC therefore is able to restore the contractile machinery but not the response induced by the desensitizing agonist itself. Homologous desensitization therefore appears to involve some PKC-independent mechanisms as suggested (Ingleson et al., 1993). This hypothesis is supported by the observation that the inhibition of CCK-induced release of InsPs and Ca2+ was practically completed within the first 30 s. Neither inhibition of Ca2+ release in desensitized cells, whose extent was similar to that previously found in guinea pig pancreatic acini (Servant et al., 1995), nor the inhibition of the InsP release was critical for the greater homologous desensitization, because a similar inhibition was detected in smooth muscle cells pre-exposed for 30 s or 5 min to CCK. In smooth muscle cells, as in other cellular systems, homologous and heterologous desensitization appear to be completely independent processes (Chuang et al. 1996).

In conclusion, CCK, SP, and GRP induced a desensitization of smooth muscle cell contraction that, depending on the time of pre-exposure of the cell to the agonist, was either homologous or heterologous. After occupancy of specific membrane receptors, there is a rapid contraction that reaches its maximum after 30 s, and, simultaneously, homologous desensitization begins. Subsequently, the contractile response decreases and progression to the heterologous pattern occurs. The cell becomes refractory at first to the same agonist and then to other receptor agonists. Thirty minutes later, the efficacy of the contractile cascade machinery is fully restored.

References


