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Heterologous desensitization of muscarinic receptors by P_{2Z} purinoceptors in rat parotid acinar cells

Yasue Fukushi *

Department of Physiology, Tohoku University School of Medicine, Seiryo-cho 2-1, Aobaku, Sendai 980-8575, Japan

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Abstract

We studied the heterologous desensitization of muscarinic receptors by ATP in fura-2-loaded rat parotid acinar cells. Exposure to ATP or 3′-*o*-(4-benzoyl) benzoyl-ATP shortened the duration and decreased the magnitude of acetylcholine-induced Ca^{2+} release from intracellular Ca^{2+} stores in a dose-dependent manner. The shortening was observed only in an early stage of desensitization (within 20 s), whereas the decrease in the magnitude of the response was dependent upon the time the cells were exposed to the nucleotides. Atropine induced a profound shortening during the progressive decrease in the magnitude of acetylcholine-induced Ca^{2+} release. 3′-*o*-(4-Benzoyl) benzoyl-ATP did not induce an increase in the cytosolic Ca^{2+} concentration when the cells were incubated in the Ca^{2+}- and Na^{+}-free medium, but it did induce a strong desensitization of muscarinic receptors. The specific protein kinase C inhibitor bisindoylmaleimide resensitized the 3′-*o*-(4-benzoyl) benzoyl-ATP-treated muscarinic receptors. Phorbol 12-myristate 13-acetate potentiated the desensitization of muscarinic receptors. Ceramides that prevent the activation of phospholipase D resensitized the 3′-*o*-(4-benzoyl) benzoyl-ATP-treated muscarinic receptors. These results suggest that ATP, acting through P_{2Z} purinoceptor-mediated phospholipase D, may produce a Ca^{2+}-independent protein kinase C. Heterologous desensitization of muscarinic receptors by protein kinase C may shorten the duration and decrease the magnitude of acetylcholine-induced Ca^{2+} release.

Keywords: Heterologous desensitization; P_{2Z} purinoceptor; Muscarinic receptor; Protein kinase C, Ca^{2+}-independent

1. Introduction

In autonomic nerve terminals ATP is co-stored with and possibly released with autonomic neurotransmitters (Burnstock, 1972). In rat parotid gland acini, in which there is a rich innervation of autonomic nerve fibres, extracellular ATP, through P_{2Z} purinoceptor activation, raises the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) via: (1) Ca^{2+} release from intracellular ryanodine-sensitive stores triggered by Na^{+} entry through cation-permeable channels (Fukushi et al., 1997), and (2) Ca^{2+} entry from the extracellular medium through cation-permeable channels permeable to Na^{+} and Ca^{2+} (McMillian et al., 1988; Soltoff et al., 1992). However, in rat salivary gland acini acetylcholine elevates $[Ca^{2+}]_i$ in two different ways: (1) via Ca^{2+} release from intracellular inositol 1,4,5-triphosphate-sensitive stores, and (2) through Ca^{2+} entry activated by depletion of Ca^{2+} stores (Putney, 1990; Fukushi et al., 1995, 1996). The effects of ATP on $[Ca^{2+}]_i$ raise the possibility that ATP may modify the influence of autonomic neurotransmitters on salivary gland function. Recently, it has been shown that extracellular ATP prevents the acetylcholine-induced increase in $[Ca^{2+}]_i$ in rat submandibular acinar cells, but not because of a direct effect of ATP on the interaction between the agonist and the receptors, because ATP had little effect on the displacement of radiolabeled acetylcholine from binding sites on salivary gland acini (Hurley et al., 1993). Furthermore, the effect of ATP did not seem to be caused by the stimulation of the Ca^{2+} pump on the plasma membrane or by an increase in the uptake of Ca^{2+} into intracellular stores (Hurley et al., 1993).

The mechanism by which extracellular ATP inhibits acetylcholine-induced Ca^{2+} mobilization, however, is still poorly understood (Hurley et al., 1993). One possible mechanism may be the heterologous desensitization of muscarinic receptors by extracellular ATP. It is widely accepted that neurotransmitter receptor desensitization is modulated by protein phosphorylation. The molecular

* Tel.: +81-22-717-8070; Fax: +81-22-717-8098; E-mail: yasue-f@mail.cc.tohoku.ac.jp

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mechanisms underlying rapid desensitization imply an alteration in the functioning of the receptor. The addition of the highly charged phosphate molecule to the receptor protein changes the conformation of the receptor protein and thereby changes the functional properties of the receptor (Huganir and Greengard, 1990). Protein phosphorylation is effected by a variety of protein kinases including members of the protein kinase C family (Huganir and Greengard, 1990).

Extracellular ATP causes potent and sustained phosphorylation (Exton, 1994). There is evidence that extracellular ATP, as a result of P_{2Z} purinoceptor activation, stimulates the rapid hydrolysis of phosphatidylcholine in large amounts via activation of phospholipase D (El-Moatassim and Dubyak, 1992), producing diacylglycerol and phosphatidic acid (Exton, 1994). A sustained elevation of diacylglycerol levels is responsible for the initiation of protein kinase C (Nishizuka, 1986). In addition, a recent in vitro study has shown that phosphatidic acid, but not diacylglycerol, induces phosphorylation of a wide range of proteins, activating a novel Ca^{2+}-independent protein kinase C (Waite et al., 1997).

Therefore, in the present study we aimed to examine (1) the process by which ATP causes desensitization of the acetylcholine-induced increase in $[Ca^{2+}]_i$ and (2) the protein kinase C responsible for the inhibitory action of ATP. Here, we provide evidence that extracellular ATP, acting through P_{2Z} purinoceptors, may cause phospholipase D production. It is further suggested that phospholipase D may produce a Ca^{2+}-independent protein kinase C, which, in turn, may cause heterologous desensitization of muscarinic receptors. As a result, the duration and magnitude of the acetylcholine-induced release of Ca^{2+} from intracellular Ca^{2+} stores is decreased.

2. Materials and methods

2.1. Solutions and materials

The standard external solutions contained 140 mM NaCl, 4.2 mM KCl, 1.13 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES–NaOH (pH 7.2). To make a Ca^{2+}-free solution, $CaCl_2$ was removed and EGTA was added to adjust the final concentration to 0.5 mM. Na^+-free solutions were prepared by replacing NaCl by *N*-methyl-D-glucamine, and the pH was adjusted to 7.2 using HCl. Reagents were obtained from Sigma (St. Louis, MO) except for fura-2/AM (Dojin Chemical Institute, Fukuoka) and collagenase (Wako, Osaka). Pretreatment of the cells with agents was performed at 37°C for 10 min [16 nM phorbol 12-myristate 13-acetate, 3 or 0.3 μM bisindoylmaleimide and 50 μM ceramides]. The substances were eliminated from the bathing solution by washing the cells several times with the standard solution before Ca^{2+} was measured.

2.2. Cell isolation and fura-2 loading

Parotid acinar cells from male Wistar rats were prepared as described previously (Fukushi et al., 1997). Briefly, parotid glands were minced and digested with

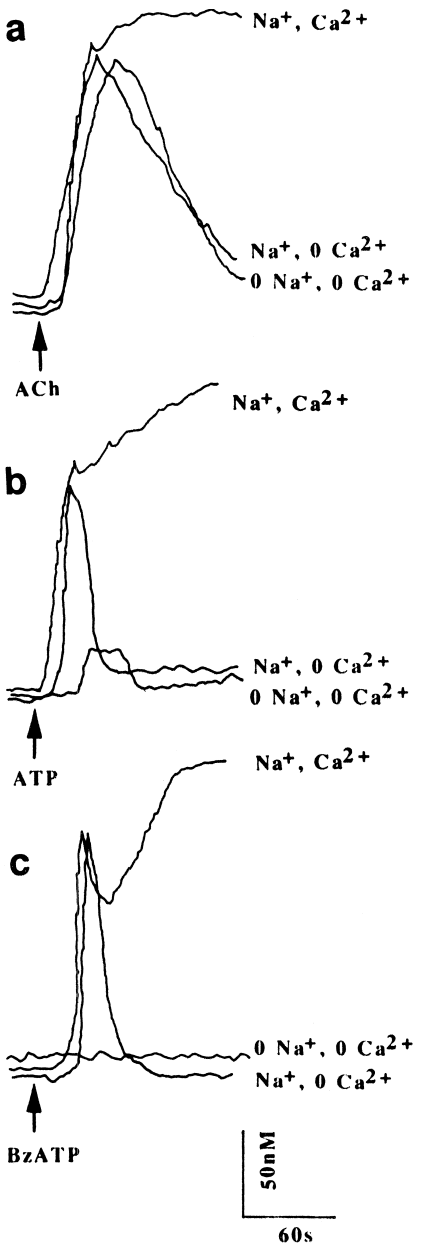

Fig. 1. Effects of Na^+ and/or Ca^{2+} removal from the external solution on the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) evoked by acetylcholine, ATP and 3′-*o*-(4-benzoyl) benzoyl-ATP. Na^+, Ca^{2+}: standard external solution containing 140 mM Na^+ and 1 mM Ca^{2+}. Na^+, 0 Ca^{2+}: high Na^+ and EGTA-containing external solution ($[Ca^{2+}]_o = 1$ nM). 0 Na^+, 0 Ca^{2+}: Na^+-free and EGTA-containing external solution. (a) Acetylcholine at 300 nM was added as indicated. (b) ATP at 2 mM was added as indicated. (c) 3′-*o*-(4-Benzoyl) benzoyl-ATP at 10 μM (adjusted to be equipotent to 2 mM ATP) was added as indicated. Note that the increase in $[Ca^{2+}]_i$ induced by ATP, or 3′-*o*-(4-benzoyl) benzoyl-ATP was markedly reduced by omitting external Na^+. The records shown are typical of three independent experiments.

collagenase (200 U/ml) for 50 min at 37°C. At an interval of 25 min the collagenase was exchanged for fresh collagenase and the incubation was continued. At the end of the collagenase treatment, the cell suspension was gently pipetted up and down to further separate the cells. Single or small clusters of cells were chosen for experiments.

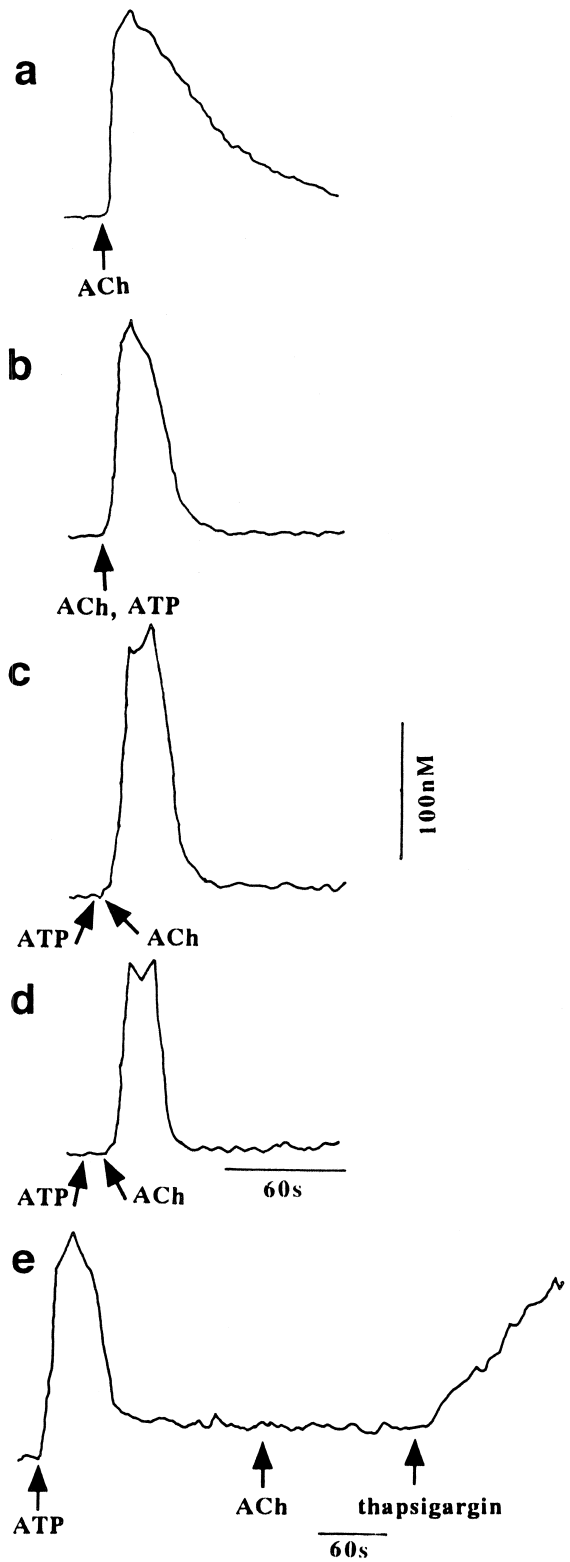

Table 1
The magnitude and duration of the acetylcholine-induced increase in $[Ca^{2+}]_i$ in cells exposed to ATP and 3́-*o*-(4-benboyl) benzoyl-ATP in various external solutions

	$\Delta [Ca^{2+}]_i$ (nM)	Duration (s)	*n*
0 Ca^{2+}, 140 mM Na^+			
ACh (300 nM)	122.6±18.9	46.5±0.5	198
ATP (2 mM)	125.5±12.2	35.1±1.9	80
0 s	118.1±3.2[ns]	31.8±1.4[a]	73
5 s	206.1±8.4[+a]	27.7±1.3[a]	19
10 s	110.4±2.1[a]	21.0±0.3[a]	74
4 min	0	0	30
0 Ca^{2+}, 0 Na^+ (NMDG)			
ACh (300 nM)	117.3±1.8	48.7±0.9	131
ATP (2 mM)	46.6±6.7	18.1±1.3	40
0 s	114.2±1.7[ns]	26.7±1.4[a]	64
5 s	75.6±5.0[a]	20.5±1.2[a]	35
10 s	63.8±1.5[a]	14.2±0.6[a]	18
2 min	0	0	40
ACh (300 nM)	117.3±1.8	48.7±0.9	131
10 μM BzATP	0	0	100
0 s	120.0±2.7[ns]	17.6±0.3[a]	35
5 s	80.3±2.7[a]	18.0±1.9[a]	23
10 s	71.3±2.2[a]	17.4±0.4[a]	28
20 s	62.8±4.5[a]	13.9±1.4[a]	23
30 s	24.0±1.2[a]	–	29
40 s	16.3±0.6[a]	–	30
60 s	0	–	100

0 Ca^{2+}: high Na^+ and EGTA-containing external solution ($[Ca^{2+}]_o = 1$ nM). 0 Na^+: Na^+ was iso-osmotically replaced by *N*-methyl-D-glucamine (NMDG). $\Delta [Ca^{2+}]_i$ was measured by subtracting the prestimulated level of $[Ca^{2+}]_i$ from the peak response. Duration was measured as the time that the increase in $[Ca^{2+}]_i$ was maintained at the level of half of the peak response. 0 s; the increase in $[Ca^{2+}]_i$ induced by the simultaneous addition of acetylcholine and a nucleotide. *t* (seconds or minutes); a nucleotide *t* (seconds or minutes) prior to the addition of acetylcholine.
[a] $P < 0.001$ less than control value.
[+a] $P < 0.001$ more than control value.
ns; not significant.
Control means the increase in $[Ca^{2+}]_i$ induced by 300 nM acetylcholine.

Cells were incubated with 1–1.5 μM fura-2/AM for 40 min at 37°C and then washed with an external solution containing 0.2% bovine serum albumin. The cells were kept at room temperature until used.

2.3. Fluorescence measurements

The coverslip with the cells was mounted in a chamber on the stage of an inverted microscope (TMD-EFQ; Nikon,

Fig. 2. The magnitude and duration of the acetylcholine-induced increase in $[Ca^{2+}]_i$ in cells exposed to ATP. (a) Acetylcholine (300 nM)-induced increase in $[Ca^{2+}]_i$. (b) An increase in $[Ca^{2+}]_i$ induced by the simultaneous addition of acetylcholine and ATP (2 mM). (c–d) ATP and acetylcholine were added as indicated. (e) ATP, acetylcholine and thapsigargin (2 μM) were added as indicated. $[Ca^{2+}]_o$ was 1 nM. Note that exposure to ATP shortened the duration and decreased the magnitude of the acetylcholine-induced increase in $[Ca^{2+}]_i$. The records shown are typical of three independent experiments using 65–131 cells.

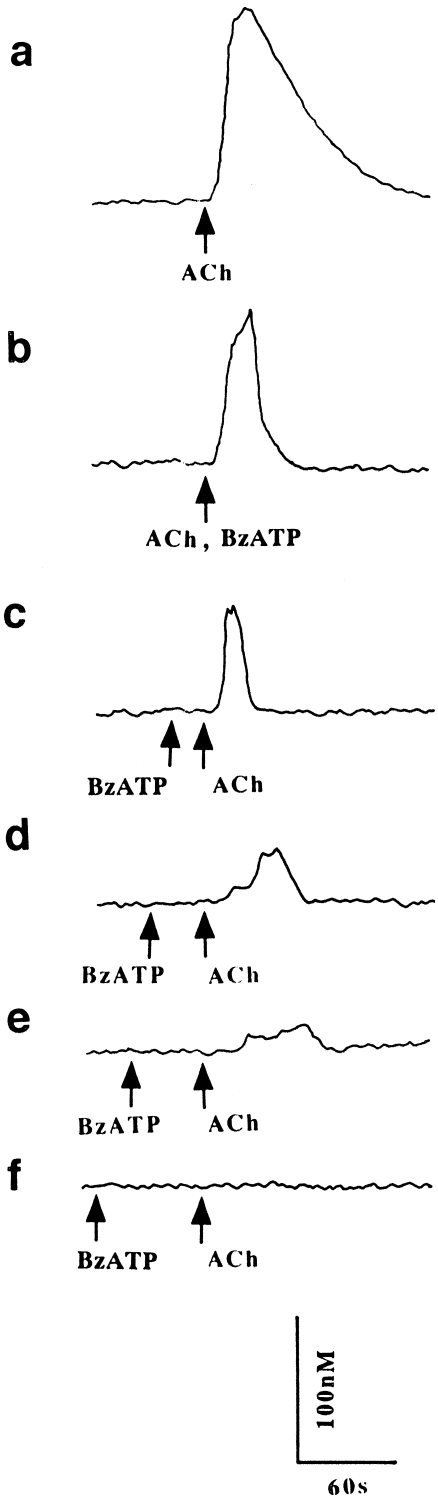

Fig. 3. The magnitude and duration of the acetylcholine-induced increase in $[Ca^{2+}]_i$ in cells exposed to 3′-*o*-(4-benzoyl) benzoyl-ATP. (a) Acetylcholine (300 nM)-induced increase in $[Ca^{2+}]_i$. (b) An increase in $[Ca^{2+}]_i$ induced by simultaneous addition of acetylcholine and 3′-*o*-(4-benzoyl) benzoyl-ATP. (c–f) 3′-*o*-(4-Benzoyl) benzoyl-ATP and acetylcholine were added as indicated. Note that exposure to 3′-*o*-(4-benzoyl) benzoyl-ATP for 60 s completely desensitized the muscarinic receptors. $[Ca^{2+}]_o$ was 1 nM and $[Na^+]_o$ was zero. The records shown were typical of three independent experiments using 32–131 cells.

Tokyo, Japan), and the cells were perfused continuously (bath volume = 400 μl; flow rate = 0.5 ml/min) with a stream of external solution at room temperature. The arrangement of the superfusion system permitted rapid exchange of the bath solution in the vicinity of the cell. Fura-2 imaging, construction of ratio images, and quantification were performed as previously described (Fukushi et al., 1995, 1997). The $[Ca^{2+}]_i$ was determined from the ratio of fluorescence traces at the two excitation wavelengths (340/380 nm) (Grynkiewicz et al., 1985).

2.4. Statistics

Statistical significance was analyzed with Student's *t*-test. Data are expressed as means ± S.E.M.

3. Results

3.1. Increase in $[Ca^{2+}]_i$ induced by acetylcholine, ATP, or 3′-o-(4-benzoyl) benzoyl-ATP under various conditions

When exposed to 300 nM acetylcholine (maximally effective concentration), the rat parotid acinar cells showed a rapid increase in $[Ca^{2+}]_i$ followed by a steady state elevation of $[Ca^{2+}]_i$ when the external solution contained 1

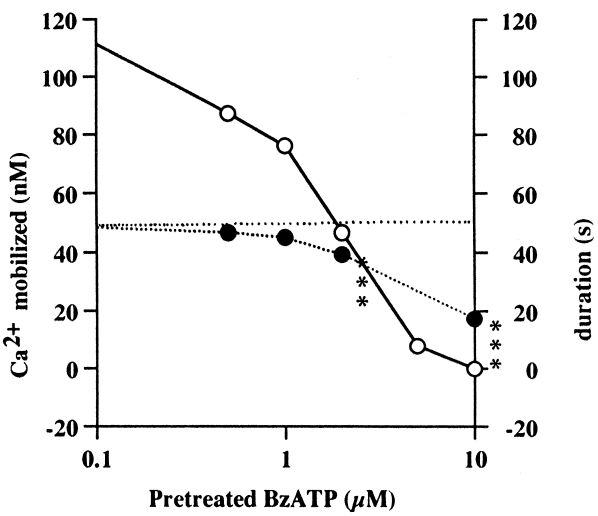

Fig. 4. Concentration-dependent inhibition of the duration and magnitude of the acetylcholine-induced increase in $[Ca^{2+}]_i$ by 3′-*o*-(4-benzoyl) benzoyl-ATP. To study the magnitude of the response, 3′-o-(4-benzoyl) benzoyl-ATP at various concentrations was added 60 s prior to the addition of acetylcholine (300 nM). To study the duration of the response, 3′-*o*-(4-benzoyl) benzoyl-ATP was added 10 s prior to the addition of acetylcholine (300 nM) in the Na^+-free external solution containing 1 nM Ca^{2+}. The amount of Ca^{2+} mobilized (open circles) was measured by subtracting the prestimulation level of $[Ca^{2+}]_i$ from the peak response. The response duration (closed circles) was measured as the time the increase in $[Ca^{2+}]_i$ was maintained at the level of half of the peak response. The duration of the acetylcholine-induced increase in $[Ca^{2+}]_i$ without application of 3′-*o*-(4-benzoyl) benzoyl-ATP (control response) is indicated by the dashed line. Each point represents the mean ± S.E.M. of three experiments using 32–131 cells. $***P < 0.001$.

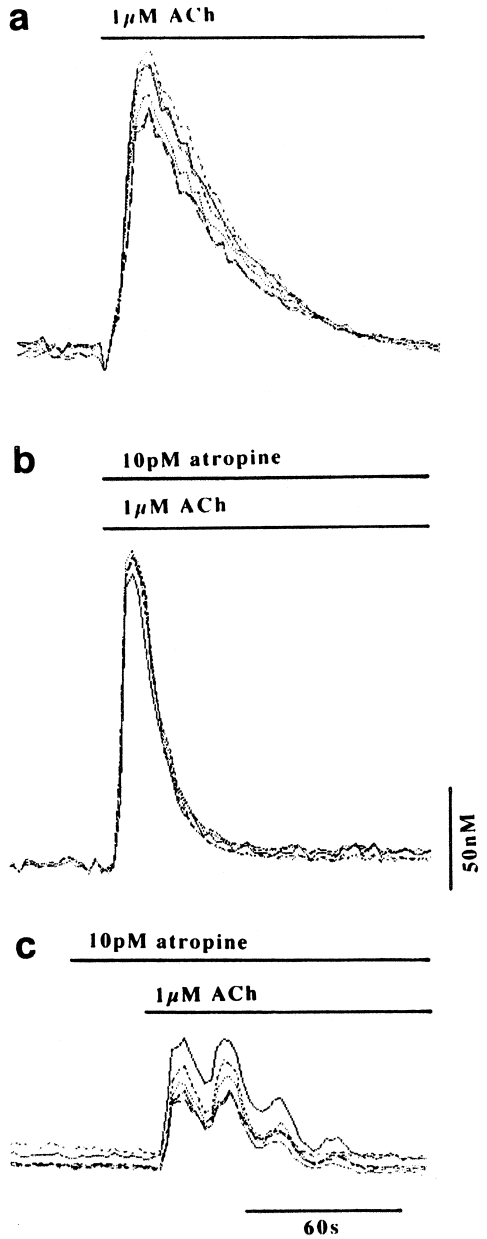

Fig. 5. Effects of atropine on duration and magnitude of the acetylcholine-induced increase in $[Ca^{2+}]_i$. (a) The increase in $[Ca^{2+}]_i$ induced by a supramaximal dose of acetylcholine (1 μM). (b) The increase in $[Ca^{2+}]_i$ induced by the simultaneous addition of acetylcholine and atropine (10 pM). (c) Atropine and acetylcholine were added as indicated. Note that shortening of the response duration occurred only at an early stage of the receptor-block, whereas the decrease in the response magnitude was time dependent. The records shown are typical of three independent experiments using 28–42 cells.

mM Ca^{2+} (Fig. 1a; Na^+, Ca^{2+}). When the external Ca^{2+} concentration was reduced to 1 nM, acetylcholine caused a transient increase in $[Ca^{2+}]_i$ (Fig. 1a; Na^+, 0 Ca^{2+}), which was unaffected by the removal of external Na^+ (Fig. 1a; 0 Na^+, 0 Ca^{2+}).

When exposed to 2 mM ATP (maximally effective concentration) or 10 μM 3′-*o*-(4-benzoyl) benzoyl-ATP (adjusted to be equipotent to 2 mM ATP), a selective agonist of the P_{2Z} purinoceptor, cells showed a rapid increase in $[Ca^{2+}]_i$ followed by a steady state elevation of $[Ca^{2+}]_i$ when the external solution contained 1 mM Ca^{2+} (Fig. 1b or c; Na^+, Ca^{2+}). When the external Ca^{2+} concentration was reduced to 1 nM, ATP caused a transient increase in $[Ca^{2+}]_i$ (Fig. 1b or c; Na^+, 0 Ca^{2+}), which was markedly reduced by the removal of external Na^+ (Fig. 1b; 0 Na^+, 0 Ca^{2+}). In the case of 3′-*o*-(4-benzoyl) benzoyl-ATP, the increase in $[Ca^{2+}]_i$ was abolished by Na^+ removal (Fig. 1c; 0 Na^+, 0 Ca^{2+}) (Fukushi et al., 1997).

3.2. Exposure to ATP shortens the duration and decreases the magnitude of the acetylcholine-induced increase in $[Ca^{2+}]_i$

As shown in Fig. 2a, acetylcholine at 300 nM induced an increase in $[Ca^{2+}]_i$ in the EGTA-containing external solution (control response). When acetylcholine and ATP (2 mM) were added simultaneously, the increase in $[Ca^{2+}]_i$ immediately reached a peak, and then decreased sharply, resulting in a shorter response (Fig. 2b). The magnitude of the response was nearly as large as that induced by acetylcholine alone, but smaller than the sum of the responses to each agent administered separately. When ATP was added 5 s prior to the addition of acetylcholine, the increase in $[Ca^{2+}]_i$ was suddenly augmented to 174% compared to the increase in $[Ca^{2+}]_i$ when ATP and acetylcholine were added simultaneously (Fig. 2c), a response which was abolished in the absence of external Na^+ (see Table 1). However, exposure to ATP thereafter decreased the magnitude of the response in a time-dependent manner (Fig. 2d). This Na^+-dependent increase in $[Ca^{2+}]_i$ confirmed our previous findings that ATP induces a Na^+-dependent in-

Table 2
Relationship between the magnitude and duration of the acetylcholine-induced increase in $[Ca^{2+}]_i$, in the presence or absence of atropine

ACh (nM)	Δ $[Ca^{2+}]_i$ (nM)	Duration (s)	*n*
10	27.9 ± 5.3	47.2 ± 0.2	14
30	34.9 ± 8.8	50.6 ± 0.2	194
100	92.4 ± 21.2	48.3 ± 1.1	138
300	122.6 ± 18.9	46.5 ± 0.5	198
1000	118.1 ± 4.7	32.2 ± 0.8[a]	70
300, atr, 0 s	62.5 ± 3.7[a]	25.5 ± 0.7[a]	42
300, atr, 30 s	0	0	42
1000, atr, 0 s	127.2 ± 14.6[ns]	18.5 ± 0.7[a]	28
1000, atr, 30 s	63.0 ± 0.9[a]	29.7 ± 0.9[+a]	42

300, atr, 0 s: the increase in $[Ca^{2+}]_i$ induced by the simultaneous addition of 300 nM acetylcholine and 10 pM atropine. 300, atr, 30 s: 10 pM atropine was added 30 s prior to the addition of 300 nM acetylcholine. 1000, atr, 0 s: the increase in $[Ca^{2+}]_i$ induced by the simultaneous addition of 1 μM acetylcholine and 10 pM atropine. 1000, atr, 30 s: 10 pM atropine was added 30 s prior to the addition of 1 μM acetylcholine.

[a] $P < 0.001$ less than control value.

Control means the increase in $[Ca^{2+}]_i$ induced by 300 nM or 1 μM acetylcholine.

[+a] $P < 0.001$ more than the value of 1000, atr, 0 s.

crease in $[Ca^{2+}]_i$ (Fukushi et al., 1997). The decrease in the magnitude of the response is due to purinoceptor activation but does not result from the emptying of Ca^{2+} stores, which were readily mobilized by thapsigargin in the presence of ATP (Fig. 2e).

3.3. Ca^{2+}-independent inhibition of the acetylcholine-induced increase in $[Ca^{2+}]_i$ by P_{2Z} purinoceptor activation

As shown in Fig. 1c (0 Na^+, 0 Ca^{2+}), 3′-*o*-(4-benzoyl) benzoyl-ATP at 10 μM did not induce an increase in $[Ca^{2+}]_i$ when the cells were incubated in the Na^+- and Ca^{2+}-free medium. However, 3′-*o*-(4-benzoyl) benzoyl-ATP had a profound effect on the acetylcholine-induced increase in $[Ca^{2+}]_i$. When 3′-*o*-(4-benzoyl) benzoyl-ATP and acetylcholine were added simultaneously, the duration of the increase in $[Ca^{2+}]_i$ induced by acetylcholine was markedly shorter without there being a significant decrease in the magnitude of the response (Fig. 3b). As shown in Fig. 2b–e, the shortening of the response duration was induced by a 10–20 s exposure to the nucleotide, whereas the decrease in the magnitude of the response was dependent on the time the cells were exposed to the nucleotides. Exposure to 3′-*o*-(4-benzoyl) benzoyl-ATP for 60 s completely abolished the increase in $[Ca^{2+}]_i$ induced by acetylcholine, in a Ca^{2+}-independent manner. The effects of ATP and 3′-*o*-(4-benzoyl) benzoyl-ATP on the acetylcholine-induced $[Ca^{2+}]_i$ in the various external medium are summarized in Table 1.

Fig. 4 shows the relationship between the concentration of 3′-*o*-(4-benzoyl) benzoyl-ATP and the magnitude and duration of the acetylcholine-induced increase in $[Ca^{2+}]_i$. As shown, the magnitude and duration of the response were clearly dependent on the concentration of 3′-*o*-(4-benzoyl) benzoyl-ATP. 3′-*o*-(4-Benzoyl) benzoyl-ATP-treated cells became resensitized to acetylcholine after they had been extensively washed with external solution for 10 min.

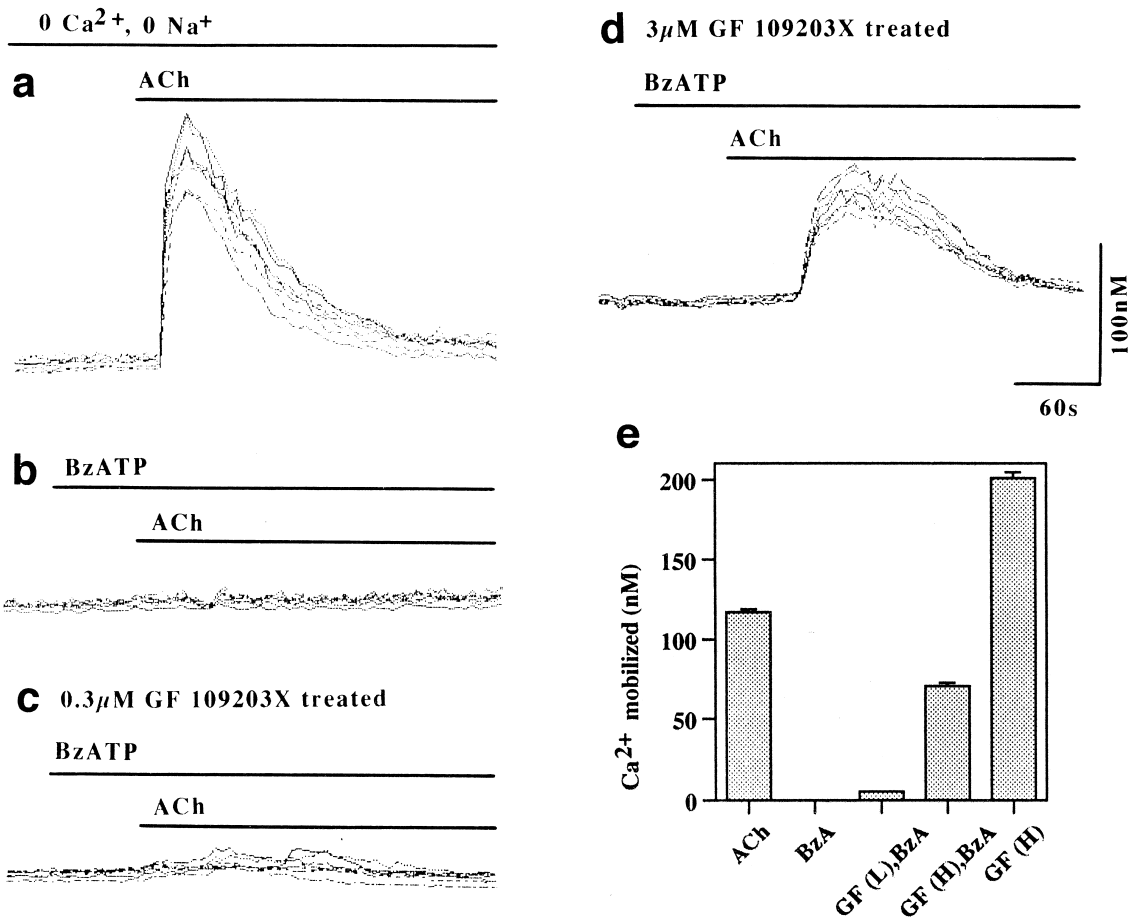

Fig. 6. Effects of the specific blocker of protein kinase C on muscarinic receptors desensitized by exposure of cells to 3′-*o*-(4-benzoyl) benzoyl-ATP. (a) The acetylcholine (300 nM)-induced increase in $[Ca^{2+}]_i$. (b) Desensitized response elicited by exposure of cells to 3′-*o*-(4-benzoyl) benzoyl-ATP. (c–d) Resensitized response after pretreatment of cells with bisindolylmaleimide (GF 109203 X) at 0.3 μM (c) and 3 μM (d). Cells were perfused with the Na^+-free external solution containing 1 nM Ca^{2+}. (e) Increase in $[Ca^{2+}]_i$ induced by acetylcholine in 3′-*o*-(4-benzoyl) benzoyl-ATP-treated cells treated [GF(L),BzA, 0.3 μM; GF(H),BzA, 3 μM] or not treated with [ACh, BzA] bisindolylmaleimide. ACh; control response induced at 300 nM. BzA; desensitized response induced by exposure of cells to 3′-*o*-(4-benzoyl) benzoyl-ATP. GF(H); control response induced by acetylcholine in cells treated with 3 μM bisindolylmaleimide. Each value represents the mean ± S.E.M. of three experiments using 28–42 cells.

3.4. Effects of atropine on time course and magnitude of the acetylcholine-induced increase in $[Ca^{2+}]_i$

The present study addresses the question of how desensitization, during which there is a functional depression of muscarinic receptors, affects the magnitude and duration of the increase in $[Ca^{2+}]_i$ induced by acetylcholine. To answer this, atropine was used instead of nucleotides. Fig. 5a shows the increase in $[Ca^{2+}]_i$ induced by 1 μM acetylcholine. When acetylcholine and 10 pM atropine were added simultaneously, the duration of the increase in $[Ca^{2+}]_i$ induced by acetylcholine was markedly shorter without there being a significant decrease in the magnitude of the response (Fig. 5b). However, the magnitude of the response progressively decreased as the exposure time increased, whereas the duration of the response was no longer affected (Fig. 5c).

Addition of acetylcholine alone to cells resulted in a dose-dependent increase in the average $[Ca^{2+}]_i$ (Table 2). The maximum increase in $[Ca^{2+}]_i$ was obtained with 300 nM acetylcholine and did not increase with a higher acetylcholine concentration (1 μM). In contrast, addition of acetylcholine to the cells, in concentrations up to 300 nM, did not result in a dose-dependent change in the duration of the response. However, 1 μM acetylcholine shortened significantly the duration of the increase in $[Ca^{2+}]_i$, and apparently reduced the magnitude of the increase (Table 2).

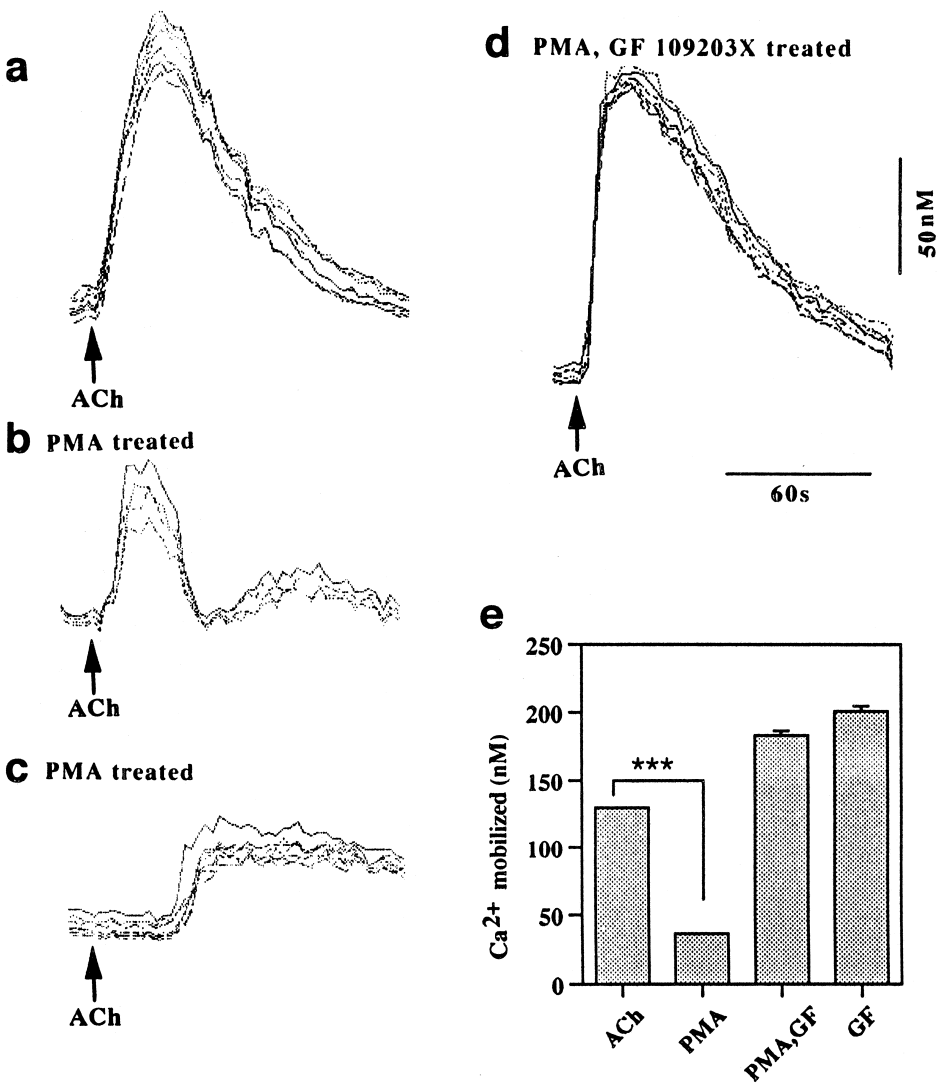

Fig. 7. Effects of a protein kinase C activator on the acetylcholine-induced increase in $[Ca^{2+}]_i$. (a) The acetylcholine-induced increase in $[Ca^{2+}]_i$ in cells pretreated with 16 nM phorbol 12-myristate 13-acetate. The response duration varied in cells treated with the protein kinase C activator. Transient type (b) and prolonged type (c). (d) The acetylcholine-induced increase in $[Ca^{2+}]_i$ in cells treated with phorbol 12-myristate 13-acetate and then with bisindolylmaleimide (GF 109203 X). $[Ca^{2+}]_o$ was 1 nM. Acetylcholine at 300 nM was added as indicated. (e) Increase in $[Ca^{2+}]_i$ induced by acetylcholine in cells treated (PMA,GF, GF) or not treated (ACh, PMA) with the protein kinase C inhibitor. Each value represents the mean ± S.E.M. of three experiments using 142–198. $***P < 0.001$.

The relationship between duration and magnitude of the increase in $[Ca^{2+}]_i$ induced by acetylcholine in the presence and absence of atropine is summarized in Table 2.

3.5. Protein kinase C inhibitor resensitizes the 3′-o-(4-benzoyl) benzoyl-ATP-treated muscarinic receptors

Fig. 6a shows a typical $[Ca^{2+}]_i$ response induced by acetylcholine in the Na^+-free and EGTA-containing external solution. Exposure to 3′-*o*-(4-benzoyl) benzoyl-ATP for 60 s completely blocked the acetylcholine response (Fig. 6b).

After pretreatment with the specific protein kinase C inhibitor bisindolylmaleimide, the muscarinic receptors became resensitized, in a dose-dependent manner (Fig. 6c–d). Before exposure to 3′-*o*-(4-benzoyl) benzoyl-ATP, the acetylcholine-induced increase in $[Ca^{2+}]_i$ was 117.3 ± 1.8 nM ($n = 131$). The increase in $[Ca^{2+}]_i$ elicited by acetylcholine in the presence of 3′-*o*-(4-benzoyl) benzoyl-ATP alone and after treatment with 0.3 and 3 μM bisindolylmaleimide was 0 nM ($n = 100$), 5.6 ± 1.5 nM ($n = 49$) and 70.7 ± 1.9 nM ($n = 42$), respectively (Fig. 6e). Before exposure to 3′-*o*-(4-benzoyl) benzoyl-ATP, the duration of the acetylcholine-induced increase in $[Ca^{2+}]_i$ was 48.7 ± 0.9 s ($n = 131$). The duration of the increase in $[Ca^{2+}]_i$ elicited by acetylcholine in the presence of 3′-*o*-(4-benzoyl) benzoyl-ATP after treatment with 3 μM bisindolylmaleimide was 49.3 ± 1.6 s. Another protein kinase C inhibitor, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), at 10 μM also resensitized the 3′-*o*-(4-benzoyl) benzoyl-ATP-treated muscarinic receptors (data not shown).

Fig. 7 shows the effect of pretreatment with phorbol 12-myristate 13-acetate (PMA), a powerful activator of protein kinase C, on the increase in $[Ca^{2+}]_i$ induced by acetylcholine. Pretreatment of the cells with the phorbol ester reduced the magnitude of the increase in $[Ca^{2+}]_i$ significantly. The acetylcholine-induced increase in $[Ca^{2+}]_i$ before and after treatment with phorbol 12-myristate 13-acetate was 129.4 ± 1.3 nM ($n = 94$) and 36.5 ± 1.5 nM ($n = 142$, $P < 0.001$), respectively (Fig. 7e). The phorbol ester induced a variable change in the duration of the increase in $[Ca^{2+}]_i$, causing a transient (Fig. 7b) or prolonged response (Fig. 7c). However, the average duration of the acetylcholine-induced increase in $[Ca^{2+}]_i$ before and after treatment with the phorbol ester was not significantly different: it was 46.5 ± 0.5 s ($n = 94$) and 49.8 ± 2.3 s ($n = 142$), respectively. In cells treated first with the phorbol ester and then with the protein kinase C inhibitor, the effect of phorbol ester was cancelled out (Fig. 7d).

3.6. Ceramides that prevent the activation of phospholipase D resensitize 3′-o-(4-benzoyl) benzoyl-ATP-treated muscarinic receptors

In order to examine whether the activation of phospholipase D by P_{2Z} purinoceptors may be involved in this heterologous desensitization, ceramides that prevent the activation of phospholipase D (Abouslham et al., 1997) were used. Pretreatment with ceramides resensitized the muscarinic receptors desensitized by exposure to 3′-*o*-(4-benzoyl) benzoyl-ATP (Fig. 8). Before application of 3′-*o*-(4-benzoyl) benzoyl-ATP, the acetylcholine-induced increase in $[Ca^{2+}]_i$ was 117.3 ± 1.8 nM ($n = 131$). The acetylcholine-induced increase in $[Ca^{2+}]_i$ before and after treatment with ceramides was 0 nM ($n = 100$) and $66.2 \pm$

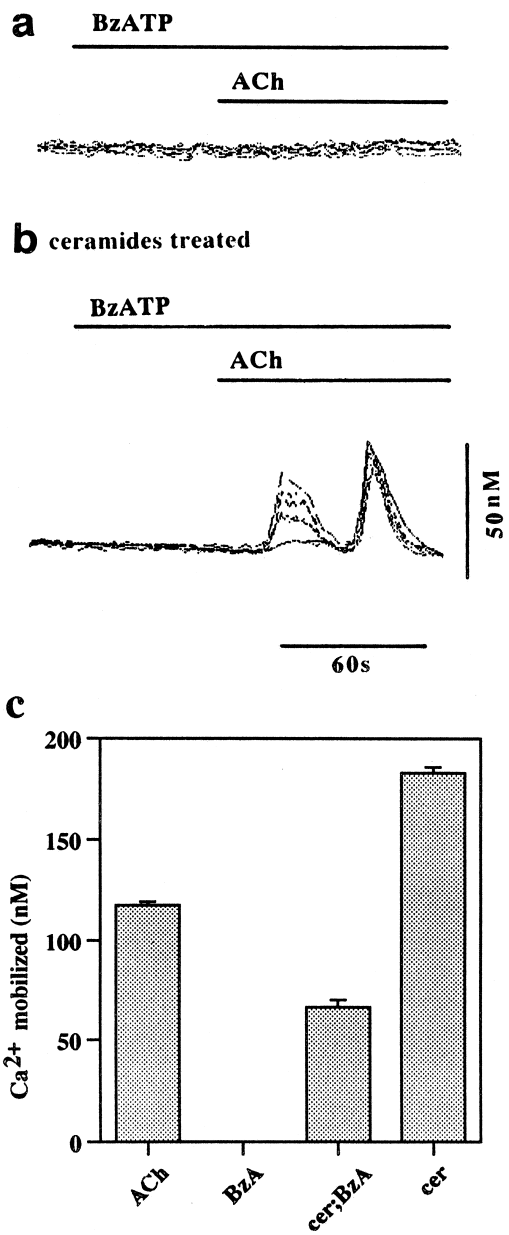

Fig. 8. Effects of ceramides on muscarinic receptors desensitized by exposure of cells to 3′-*o*-(4-benzoyl) benzoyl-ATP. (a) Desensitized response elicited by exposure of cells to 3′-*o*-(4-benzoyl) benzoyl-ATP. (b) Resensitized response after pretreatment of cells with 50 μM ceramide. $[Ca^{2+}]_o$ was 1 nM and $[Na^+]_o$ was zero. (c) Increase in $[Ca^{2+}]_i$ induced by acetylcholine in cells treated (cer,BzA; cer) or not treated (ACh, BzA) with ceramide. Each value represents the mean ± S.E.M. of three experiments using 49–131 cells.

3.7 nM ($n = 49$, $P < 0.001$), respectively (Fig. 8c). The duration of the acetylcholine-induced increase in $[Ca^{2+}]_i$ before and after treatment with ceramides was 48.7 ± 0.9 s ($n = 131$) and 15.6 ± 0.6 s ($n = 49$, $P < 0.001$), respectively.

4. Discussion

The major findings of the present investigation are that: (1) P_{2Z} purinoceptor activation decreases the duration and magnitude of the acetylcholine-induced release of Ca^{2+} from intracellular stores; (2) protein kinase C is responsible for the decreased duration and magnitude of the response; (3) the activation of protein kinase C is not required for Ca^{2+} influx or elevation of $[Ca^{2+}]_i$; and (4) phospholipase D may be involved in the activation of protein kinase C.

4.1. ATP acutely desensitizes muscarinic receptors in two distinct manners

The present finding that blockage of muscarinic receptors by atropine mimics the effects of ATP on the acetylcholine-induced increase in $[Ca^{2+}]_i$ (Fig. 5) strongly suggests that ATP acutely desensitizes muscarinic receptors. Exposure to α-bungarotoxin and (+)-tubocurarine shortens the duration of the endplate currents of anti-acetylcholine esterase-treated endplates (Katz and Miledi, 1973). Numerous studies of the desensitization of nicotinic receptors to acetylcholine (Magleby and Pallotta, 1981; Magazanik et al., 1990; Giniatullin et al., 1997) have revealed that densitization may have two features, one of which is a shortening of the duration of the response while the magnitude remains unchanged. Pennefather and Quastel (1981) reported that a slight (few percent) blockade of the receptor may not affect the endplate current. The second feature is a decrease in response magnitude when most receptors are in a desensitized state for a longer time.

The present study indicates there are also two distinct features of the desensitization of muscarinic receptors to ATP. The present finding that the shortening of the response duration was observed only at an early stage of the desensitization, whereas the decrease in the response magnitude was dependent upon the time exposed cells with ATP is consistent with the results of Giniatullin et al. (1997).

4.2. Protein kinase C activation mediated by P_{2Z} purinoceptors desensitizes muscarinic receptors in a Ca^{2+}-independent manner

It is known that acetylcholine itself desensitizes muscarinic receptors to acetylcholine. Acetylcholine at a supramaximal concentration (1 μM) shortened significantly the duration of the increase in $[Ca^{2+}]_i$, and apparently reduced the magnitude of the response (Table 2). Muscarinic receptors are linked to guanine nucleotide-binding proteins, which are functionally coupled to phospholipase C. Phospholipase C leads to the generation of inositol 1,4,5-triphosphate and diacylglycerol. Diacylglycerol, in concert with the elevation in $[Ca^{2+}]_i$, activates protein kinase C (Berridge, 1987; Billah and Anthes, 1990). Phosphorylation of muscarinic receptors by protein kinase C leads to the desensitization of muscarinic receptors to cholinergic stimulation (Ansah et al., 1986; Maruyama, 1989; Willems et al., 1993; Gromada et al., 1995).

The present study shows that elimination of protein kinase C activity, by treatment with bisindolylmaleimide, resensitized the 3′-*o*-(4-benzoyl) benzoyl-ATP-treated muscarinic receptors (Fig. 6). This is interpreted to indicate that bisindolylmaleimide inhibited the activity of protein kinase C and thereby prevented the protein kinase C-dependent phosphorylation of the muscarinic receptors.

Protein kinase C was responsible for the desensitization of muscarinic receptors in the present study, and its effect was mediated by P_{2Z} purinoceptor activation. Occupation of P_{2Z} purinoceptors is accompanied by a rapid and large increase in the activation of a phosphatidylcholine-selective phospholipase D effector enzyme. In the mouse macrophage cell system, phospholipase D activity is enhanced by the addition of ATP or 3′-*o*-(4-benzoyl) benzoyl-ATP (El-Moatassim and Dubyak, 1992). The enhanced phospholipase D activity appeared to be absolutely dependent on the formation of ligand-occupied P_{2Z} purinoceptors, but not on Ca^{2+} influx or an increase in $[Ca^{2+}]_i$.

The present finding that ceramides resensitize muscarinic receptors desensitized by exposure to 3′-*o*-(4-benzoyl) benzoyl-ATP strongly suggests that phospholipase D is involved in desensitization.

In addition, phosphatidic acid, generated by phospholipase D activation, activates one or more potentially novel protein kinases, including isoforms of protein kinase C, which are Ca^{2+}-independent (Waite et al., 1997). The present study indicates that 3′-*o*-(4-benzoyl) benzoyl-ATP had a profound effect on the acetylcholine-induced increase in $[Ca^{2+}]_i$ in a Ca^{2+}-independent manner (Fig. 3). The present finding that the desensitization induced by ATP was Ca^{2+}-independent is consistent with the results of Waite et al. (1997), although the possible involvement of Ca^{2+}-dependent protein kinase C cannot be excluded.

4.3. ATP is an attractive candidate for the first messenger that regulates the muscarinic receptor

The present study suggests that ATP is an attractive candidate for the first messenger that regulates muscarinic receptor phosphorylation, since it is a possible cotransmitter with acetylcholine (Burnstock, 1972). Since ATP-in-

duced phosphorylation is not required for the elevation of $[Ca^{2+}]_i$, ATP may desensitize muscarinic receptors more rapidly than acetylcholine does.

In conclusion, the activation of P_{2Z} purinoceptors, by the activation of phospholipase D, produces a Ca^{2+}-independent protein kinase C which, in turn, may cause heterologous desensitization of muscarinic receptors. As a result, the duration and magnitude of the acetylcholine-induced increase in $[Ca^{2+}]_i$ was reduced. Shortening of the response can result in a rapid recovery of the $[Ca^{2+}]_i$ and high-frequency signalling (Giniatullin et al., 1997), which may be important for the secretory function of the salivary gland.

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