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cAMP-dependent potentiation of the Ca^{2+}-activated release of the anionic fluorescent dye, calcein, from rat parotid acinar cells

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Abstract

A recent study indicates that elevation of $[Ca^{2+}]_i$ enhances the release of calcein, an anionic fluorescent dye, from isolated exocrine acinar cells, so cytoplasmic calcein is useful for monitoring the secretion of organic anions. In this study, we investigated the effect of cAMP on the calcein release evoked by elevation of $[Ca^{2+}]_i$. Isoproterenol, forskolin and dibutyryl cyclic AMP (dbcAMP) did not induce the release of calcein from isolated parotid acinar cells, but they potentiated the carbachol-induced release of calcein. Although cytoplasmic calcein is released through an increase in $[Ca^{2+}]_i$, isoproterenol potentiated the carbachol-induced release of calcein without affecting the increase in $[Ca^{2+}]_i$ evoked by a high concentration of carbachol (10^{-6} M). Charybdotoxin, a K^+ channel blocker, inhibited both the carbachol-induced release and the potentiation by isoproterenol. However, the calcein permeation pathways mediating the carbachol-induced release and the isoproterenol-potentiated release exhibited distinct sensitivities to anion channel blockers. Our results indicate that the calcein release induced by carbachol is potentiated through an increase in intracellular levels of cAMP. Although both the Ca^{2+}-activated release and the cAMP-potentiated release may be coupled to Ca^{2+}-activated K^+ efflux, increases in both $[Ca^{2+}]_i$ and $[cAMP]_i$ may activate the calcein conduction pathway which is not activated by an increase in $[Ca^{2+}]_i$ alone.

Keywords: Calcium ion; cAMP; Fluid secretion; Calcein; Multidrug resistance protein

1. Introduction

The multidrug resistance-associated protein (MRP) is a member of the ATP-binding cassette (ABC) superfamily of transporters and is responsible for the ATP-dependent extrusion of chemotherapeutic agents, glutathione S-conjugates and organic anions (Leier et al., 1994; Muller et al., 1994; Chang et al., 1997; Keppler et al., 1997; Gao et al, 1998; Vernhet et al., 1999). Recent studies indicate that MRP transports calcein and calcein acetoxy-methyl ester (calcein-AM) from the cytoplasm to the extracellular space (Feller et al., 1995; Hollo et al., 1996; Essodaigui et al., 1998), whereas P-glycoprotein, another ABC transporter protein, transports only calcein-AM (Essodaigui et al., 1998). Thus, calcein and calcein-AM are useful for functional testing for the presence of both MRP and P-glycoprotein activity, which is of prognostic value for multidrug resistance (Dhar et al., 1998; Legrand et al., 1998). Calcein-AM is a lipophilic, non-fluorescent compound, which rapidly permeates the plasma membrane of cells. It is transformed into the highly fluorescent organic anion, calcein, upon cleavage of the ester bonds by intracellular esterases. MRP is located in the plasma membrane, and can extrude calcein from the cell against a concentration gradient (Zaman et al., 1994; Keppler et al., 1997; Essodaigui et al., 1998).

Recently, we reported that the anionic fluorescent dye, calcein, loaded into isolated parotid acinar cells, is released from the cytoplasm through an increase in $[Ca^{2+}]_i$ induced by acetylcholine receptor and α-adrenoceptor agonists, but not through an increase in $[cAMP]_i$ induced by β-adrenoceptor agonists (Sugita et al., 1995). Salivary glands have been extensively used as a model system for fluid and electrolyte secretion by secretory epithelial cells (Foskett

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and Melvin, 1989; Nauntofte, 1992). The central feature of the accepted model of fluid and electrolyte secretion by salivary acinar cells is transepithelial Cl^- movement as the driving force for fluid and electrolyte secretion (Petersen, 1992). Parotid acinar cells express at least three distinct Cl^- channels: ClC-2-like, volume sensitive, and Ca^{2+}-activated Cl^- channels (Arreola et al., 1996). Ca^{2+}-activated Cl^- channels are believed to be the main Cl^- exit pathway in the luminal membrane, coupled to K^+ exit via a basolateral Ca^{2+}-activated K^+ channel (Soltoff et al., 1989; Petersen, 1992; Arreola et al., 1996; Hirono et al., 1998). However, the coordinated and compartmentalized activation of various Cl^- channels is essential for fluid and electrolyte secretion from secretory epithelial cells (Kasai and Augustine, 1990; Zeng et al., 1997). Nevertheless, neither the membrane localization nor regulation of various anion channels, while the fluid is being secreted and during the post-stimulation recovery period, is known. Calcein is an anionic substance whose molecular weight is 623 and which holds an electric charge of -4 or -5 in living cells. In the present study, the transient release of calcein induced by carbachol was inhibited by suppression of basolateral K^+ efflux and by depolarization, suggesting that anion channels are transiently activated by carbachol and conduct anionic calcein down an electrochemical gradient. These observations imply that anionic calcein loaded into the cytoplasm is a new tool for characterizing the anion permeation pathways during Ca^{2+}-activated fluid secretion from epithelial cells.

Extensive evidence suggests that Ca^{2+} and cAMP commonly cross-talk and modulate each other (Tsunoda, 1993). Ca^{2+} mobilizing agonists trigger several forms of $[Ca^{2+}]_i$ signals, which include $[Ca^{2+}]_i$ oscillations and Ca^{2+} waves (Foskett and Melvin, 1989; Berridge, 1993; Lee et al., 1997a,b). cAMP can modulate $[Ca^{2+}]_i$ signals by either stimulating or inhibiting Ca^{2+} channels and Ca^{2+}-ATPases (Rubin and Adolf, 1994; Chatton et al., 1998; Tertyshnikova and Fein, 1998; Wojcikiewicz and Luo, 1998). In salivary glands, cAMP potentiates Ca^{2+}-activated K^+ and Cl^- conductances, resulting in the potentiation of Ca^{2+}-induced fluid secretion (Ishikawa, 1997; Hirono et al., 1998). However, it is not clear whether an increase in $[cAMP]_i$ increases the open probability of Ca^{2+}-activated K^+ and Cl^- channels in either a $[Ca^{2+}]_i$-dependent or independent manner, or whether increases in both $[Ca^{2+}]_i$ and $[cAMP]_i$ open K^+ and Cl^- channels which are not activated by an increase in $[Ca^{2+}]_i$ alone. Anionic calcein loaded into the cytoplasm may be useful to probe the cAMP modulation of Ca^{2+}-activated electrolyte secretion from epithelial cells and to clarify the mechanism by which cAMP regulates Ca^{2+}-activated events.

In the present study, we examined the effect of cAMP-elevating agents on calcein release for which elevation of $[Ca^{2+}]_i$ is the primary regulator. Our results suggest that the calcein release enhanced by carbachol, a Ca^{2+} mobilizing agonist, is potentiated through an increase in intracellular levels of cAMP. The pharmacological properties and $[Ca^{2+}]_i$ dependence suggest that the cAMP-dependent potentiation of calcein release may be associated with activation of a distinct pathway from the carbachol-activated one, although both carbachol-induced release and isoproterenol-potentiated release may be coupled with Ca^{2+}-activated K^+ efflux. Thus, increases in both $[Ca^{2+}]_i$ and $[cAMP]_i$ activate the calcein conduction pathway which is not activated by an increase in $[Ca^{2+}]_i$ alone, and calcein is conducted down an electrochemical gradient. The calcein conduction pathway, which is sustainedly activated by increases in $[Ca^{2+}]_i$ and $[cAMP]_i$, may be useful to improve chemotherapeutic drug uptake by epithelial cells.

2. Materials and methods

2.1. Solutions and chemicals

The media used were based on Krebs–Henseleit Ringer (KHR) solution containing 103 mM NaCl, 4.7 mM KCl, 2.56 mM $CaCl_2$, 1.13 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.15 mM NaH_2PO_4, 2.8 mM glucose, 4.9 mM sodium pyruvate, 2.7 mM sodium fumarate, 4.9 mM sodium glutamate, and 0.1% bovine serum albumin, buffered with 12.5 mM HEPES at pH 7.4. The solution was thoroughly gassed with 95% O_2 and 5% CO_2 before each experiment.

Carbachol (carbamylcholine chloride), forskolin, charybdotoxin, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), glibenclamide, and N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate, sodium salt (dbcAMP) were purchased from Sigma (St. Louis, MO, USA). Diphenylamine-2-carboxylate (DPC) and A23187 were purchased from Wako (Osaka, Japan). Fura-2/AM and calcein-AM were purchased from Dojindo (Kumamoto, Japan) and Molecular Probes (Eugene, OR), respectively. DL-isoproterenol hydrochloride and DL-propranolol hydrochloride were obtained from Nacalai Tesque (Kyoto, Japan).

2.2. Measurement of calcein release from isolated acinar cells

Male Wistar rats (about 300 g) were anesthetized with sodium pentobarbital. The animals were treated in accordance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences* published by the Physiological Society of Japan, and extra care was taken to avoid animal suffering. The minced parotid glands were digested for 20 min with a 0.1% solution of collagenase (260 U/ml; type S-1, Nitta Gelatin, Osaka, Japan) dissolved in a modified KHR solution. The tissue suspension was filtered through a 100-μm nylon mesh to remove tissue clumps and then through a 50-μm nylon mesh to remove aggregates of acini. The cells were then filtered

with a 10-μm nylon mesh. The 10–50 μm fraction was centrifuged, and the pellet resuspended in a KHR solution was used as an isolated acinar cell fraction. The calcein release from isolated acinar cells was determined as described (Sugita et al., 1995). The acinar cells loaded with calcein were incubated in solution containing agonists at 37°C for 10 min. The suspension was centrifuged to separate the supernatant and the cell fractions. Nonidet P-40 (Sigma) was added to the supernatant and cell fraction to give a final concentration of 0.5 g/100 ml. Nonidet P-40 disrupted the cell membrane and induced the release of calcein remaining in the acinar cells. Fluorescence in each fraction was measured at an excitation wavelength of 490 nm and an emission wavelength of 510 nm with the aid of a fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The release of calcein is expressed as the fluorescence of the supernatant divided by the total initial fluorescence (fluorescence of the supernatant plus fluorescence from the isolated cells), and results were obtained from at least three samples in each experiment. The net release of calcein for 10 min evoked by agonists is expressed as the difference between the release of calcein without the agonists and that with agonists. Results are presented as means ± S.E.M. of n observations. Unless noted otherwise, statistical significance was determined using unpaired Student's t-test. P-values < 0.05 were considered statistically significant.

2.3. Measurement of $[Ca^{2+}]_i$

The measurement of $[Ca^{2+}]_i$ was carried out by using fura-2 and an image analysis system, ARGUS-50/CA (Hamamatsu Photonics, Hamamatsu, Japan). The isolated acinar cells were incubated with 2 μM fura-2/AM for 20 min and allowed to attach to cover slips coated with poly-L-lysine. A cover slip was placed on the stage of an inverted epifluorescence microscope. The chamber was perfused continuously with KHR solution. Fura-2 fluorescence images upon excitation at 340 and 360 nm were stored in a computer. The fluorescence ratio (F340/F360)

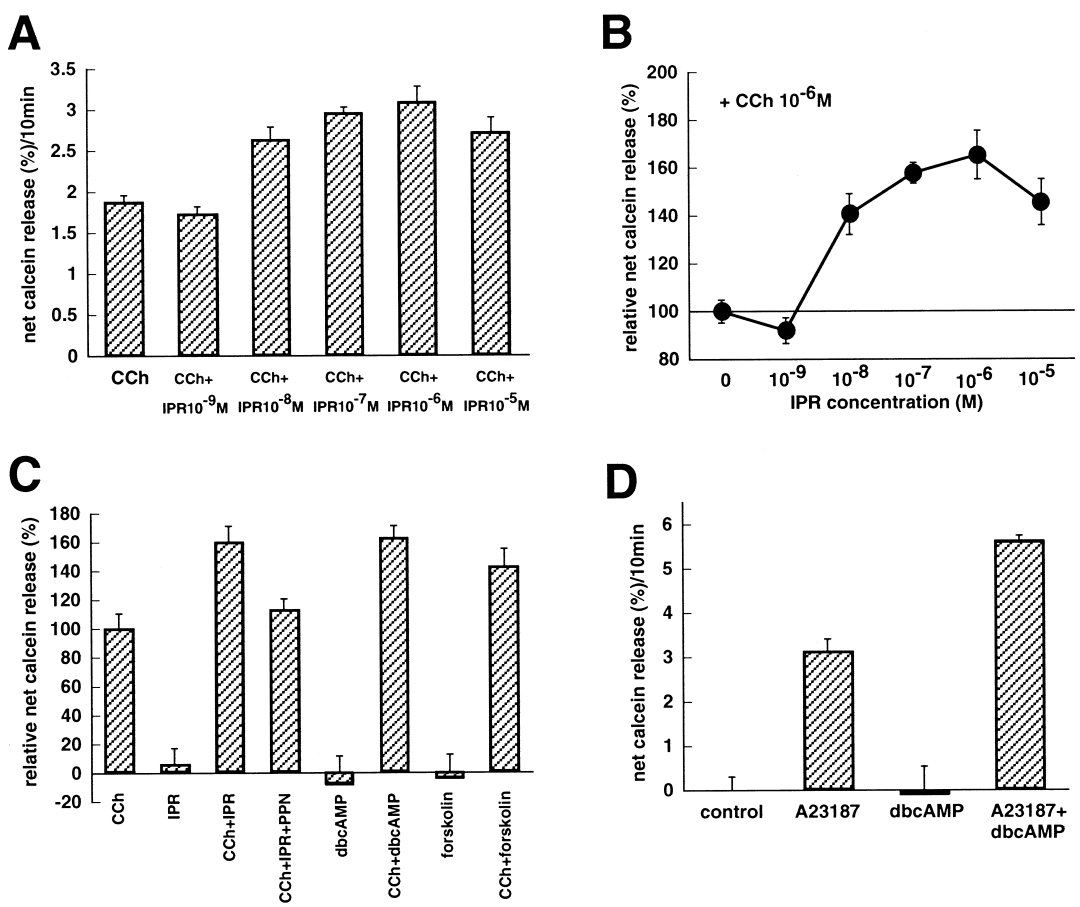

Fig. 1. An increase in $[cAMP]_i$ potentiates the carbachol (CCh)-induced release of calcein. (A) Effect of isoproterenol (IPR) on the carbachol-induced release of calcein. The net release of calcein was measured 10 min after addition of 10^{-6} M carbachol and isoproterenol at the concentrations indicated ($n = 3$). (B) A normalized dose–response curve for potentiation by isoproterenol of the carbachol-induced release of calcein. The relative net calcein release is expressed as a percentage of the net release of calcein induced by 10^{-6} M carbachol ($n = 3$). (C) Effects of isoproterenol, propranolol (PPN), dbcAMP and forskolin on the carbachol-induced release of calcein. 10^{-6} M isoproterenol, 10^{-6} M propranolol, 10^{-3} M dbcAMP, and 10^{-5} M forskolin were added to the medium in the presence or absence of 10^{-6} M carbachol ($n = 3$). (D) Effect of A23187 and dbcAMP on calcein release. The net release of calcein for 10 min was measured in the presence of 2.5×10^{-6} M A23187 and 10^{-3} M dbcAMP ($n = 3$).

was calculated simultaneously using the measurements from seven cells (Sugita et al., 1995; Hirono et al., 1998).

3. Results

3.1. The effect of isoproterenol, forskolin and dbcAMP on the carbachol-induced release of calcein

Addition of carbachol, an acetylcholine receptor agonist, but not isoproterenol, a β-adrenoceptor agonist, evoked the net release of calcein from acinar cells (Sugita et al., 1995). The calcein release induced by 10^{-6} M carbachol was potentiated by isoproterenol at concentrations of more than 10^{-8} M (Fig. 1A,B). Propranolol, a β-adrenoceptor antagonist, suppressed the isoproterenol potentiation of the carbachol-induced release of calcein ($P < 0.05$; Fig. 1C). Dibutyryl cyclic AMP (dbcAMP; 10^{-3} M), a membrane-permeant cAMP analog, enhanced the carbachol-induced release of calcein ($P < 0.05$; Fig. 1C), whereas dbcAMP itself had no effect on the net release of calcein. Forskolin (10^{-5} M), which activates adenylate cyclases, also potentiated the carbachol-induced release of calcein ($P < 0.05$; Fig. 1C). These results suggest that the carbachol-induced release of calcein is potentiated by an increase in $[cAMP]_i$. However, protein kinase A inhibitors, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89; 5×10^{-7} M) and (8*R*,9*S*,11*S*)-(−)-9-hydroxy-9-*n*-hexyloxy-carbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11*a*-triazadibenzo[*a*,*g*]cycloocta[*cde*]trinden-1-one (KT5720; 5×10^{-7} M), did not inhibit the potentiation by isoproterenol of the carbachol-induce release of calcein. A23187 (2.5×10^{-6} M), a calcium ionophore, but not dbcAMP (10^{-3} M), evoked calcein release from isolated acinar cells (Fig. 1D). The calcein release evoked by A23187 was also potentiated by dbcAMP ($P < 0.05$; Fig. 1D), implying that the calcein release induced through an increase in $[Ca^{2+}]_i$ is potentiated via elevation of $[cAMP]_i$.

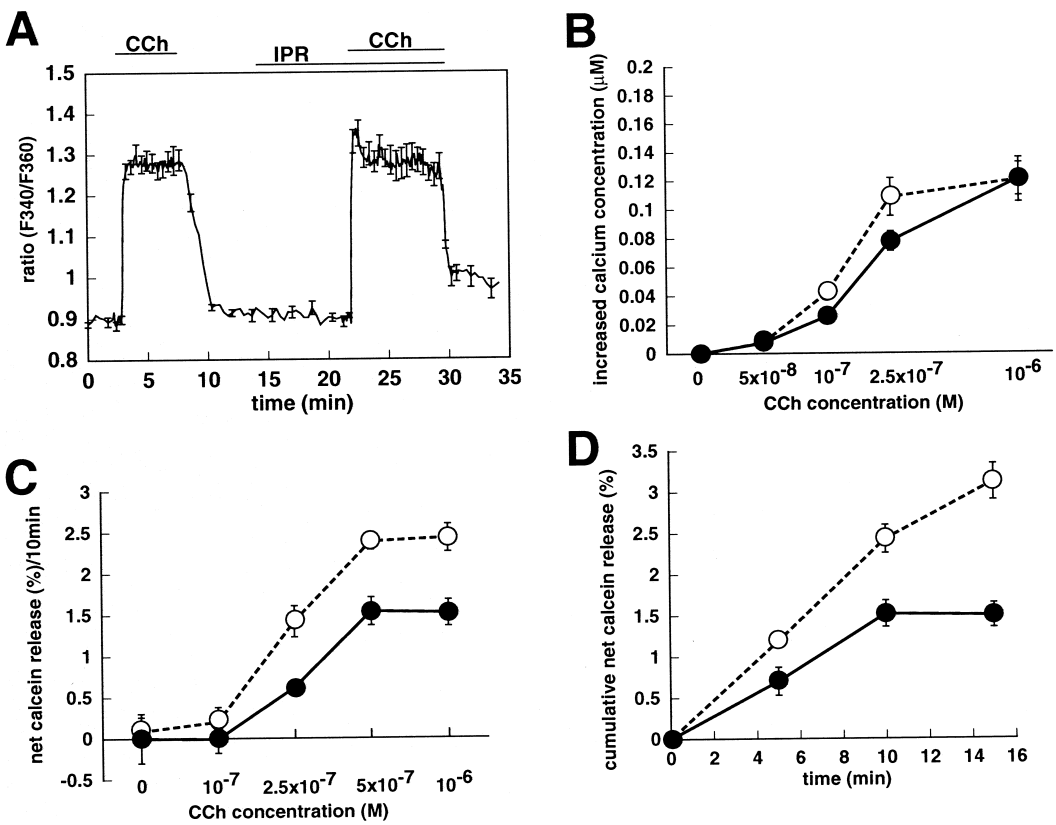

Fig. 2. Relation of $[Ca^{2+}]_i$ to cAMP-dependent potentiation of the carbachol-induced release of calcein. (A) Effect of isoproterenol (IPR) on the carbachol (CCh)-induced increase in $[Ca^{2+}]_i$. Acinar cells loaded with fura-2 were perfused with 10^{-6} M carbachol and 10^{-6} M isoproterenol during the period indicated by the horizontal bar. Changes in $[Ca^{2+}]_i$ are indicated as the mean of seven measurements (±S.E.M.) of the fluorescence ratio of the excitation at 340 nm to that at 360 nm. (B) Concentration dependence of agonist-increased $[Ca^{2+}]_i$. Changes in $[Ca^{2+}]_i$ (μM) were examined after the addition of carbachol at the concentrations indicated in the absence (closed circles) or presence (open circles) of 10^{-6} M isoproterenol ($n = 7$). (C) Concentration dependence of agonist-induced calcein release. The net release of calcein was examined after the addition of carbachol at the concentrations indicated in the absence (closed circles) or presence (open circles) of 10^{-6} M isoproterenol ($n = 3$). (D) The time course of the agonist-induced release of calcein. The cumulative net release of calcein was measured at the times indicated after the addition of 10^{-6} M carbachol (closed circles), or 10^{-6} M carbachol plus 10^{-6} M isoproterenol (open circles) ($n = 3$).

3.2. Relationship between $[Ca^{2+}]_i$ and the potentiation by isoproterenol of carbachol-induced release

The carbachol-induced release of calcein is evoked through an increase in $[Ca^{2+}]_i$. To clarify whether isoproterenol enhances the carbachol-induced release of calcein by potentiating the carbachol-induced increase in $[Ca^{2+}]_i$, we studied the effect of isoproterenol on the carbachol-induced increase in $[Ca^{2+}]_i$. Addition of carbachol at concentrations of more than 10^{-7} M induced a rapid increase in $[Ca^{2+}]_i$, which was sustained as shown in Fig. 2A. The increase in $[Ca^{2+}]_i$ induced by $1–2.5 \times 10^{-7}$ M carbachol was significantly enhanced by 10^{-6} M isoproterenol (paired *t*-test, $P < 0.05$; Fig. 2B) while isoproterenol itself did not change $[Ca^{2+}]_i$ (Fig. 2A). However, isoproterenol had little effect on the increase in $[Ca^{2+}]_i$ induced by 10^{-6} M carbachol (Fig. 2AB). The calcein release induced by carbachol at concentrations of more than 2.5×10^{-7} M was significantly enhanced by 10^{-6} M isoproterenol (Fig. 2C). Isoproterenol enhanced the calcein release induced by 10^{-6} M carbachol without affecting the sustained increase of $[Ca^{2+}]_i$ evoked by carbachol. Thus, it is likely that potentiation by 10^{-6} M isoproterenol of the calcein release induced by 10^{-6} M carbachol cannot be attributed to its effect on the cytosolic Ca^{2+} level. The time course of the cumulative net release of calcein by carbachol or carbachol plus isoproterenol is shown in Fig. 2D. The net release of calcein reached a maximum within 10 min after the addition of carbachol. However, the calcein release induced by the simultaneous addition of carbachol and isoproterenol was sustained during stimulation.

Removal of extracellular calcium ions did not affect the initial, transient increase in $[Ca^{2+}]_i$ evoked by carbachol, but abolished the sustained increase in $[Ca^{2+}]_i$ (Fig. 3A). Carbachol enhanced calcein release from acinar cells in the absence of extracellular calcium ions as much as that in the presence of extracellular calcium ions. Calcein was transiently released within 5 min after the addition of carbachol (Fig. 3B). In contrast, the potentiation of the carbachol-induced release of calcein by isoproterenol was markedly inhibited in the absence of extracellular calcium ions (Fig. 3B), while the addition of isoproterenol had little effect on the peak level of the initial, transient increase in $[Ca^{2+}]_i$ evoked by carbachol (Fig. 3A). These results suggest that the cAMP-dependent potentiation of calcein release requires a sustained increase in $[Ca^{2+}]_i$ or is regulated by Ca^{2+} entry across the plasma membrane, whereas the carbachol-induced release of calcein is triggered by a transient increase in $[Ca^{2+}]_i$ via Ca^{2+} release from intracellular stores.

3.3. The effect of ion channel blockers on the potentiation of the carbachol-induced release of calcein

Elevation of $[Ca^{2+}]_i$ stimulates fluid secretion by activating ion permeability in secretory epithelial cells (Peter-

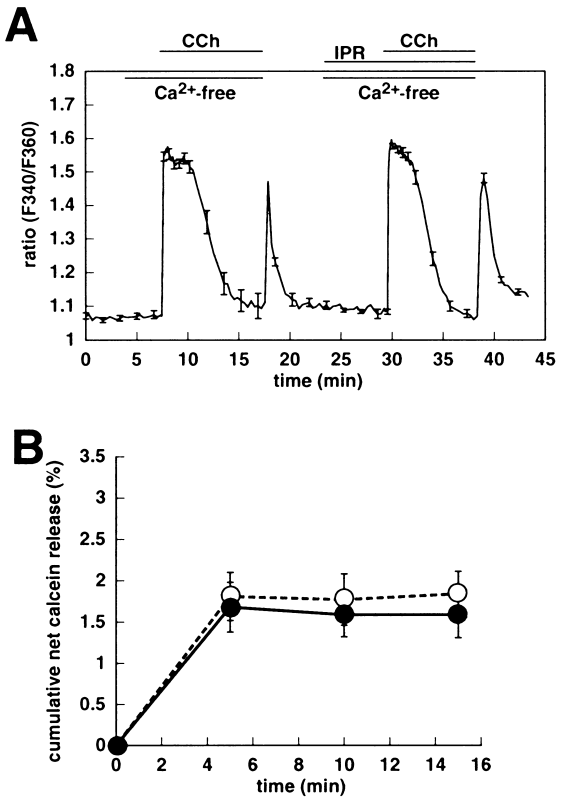

Fig. 3. Effects of Ca^{2+} removal on $[Ca^{2+}]_i$ and calcein release. (A) Changes in $[Ca^{2+}]_i$ evoked by agonists in the absence of extracellular calcium ions. Acinar cells loaded with fura-2 were perfused with 10^{-6} M carbachol (CCh) and 10^{-6} M isoproterenol (IPR) during the period indicated by the horizontal bar. The changes in $[Ca^{2+}]_i$ are presented as in Fig. 2A ($n = 7$). (B) The time course of the agonist-induced release of calcein in the absence of extracellular calcium. The cumulative net release of calcein in the absence of extracellular calcium was measured at the times indicated after the addition of 10^{-6} M carbachol (closed circles), or 10^{-6} M carbachol plus 10^{-6} M isoproterenol (open circles) ($n = 3$).

sen, 1992). Basolateral K^+ channels keep the apical (and basolateral) cell membrane potential more negative than the Nernst potential for Cl^-, thereby providing the driving force for the sustained Cl^- efflux. To clarify the involvement of Ca^{2+}-activated ion channels in calcein release, we investigated the effects of ion channel blockers. The K^+ channel blocker, 10^{-7} M charybdotoxin, markedly suppressed the net release of calcein induced by 10^{-6} M carbachol ($P < 0.05$; Fig. 4). The net release of calcein by 10^{-6} M carbachol was strongly inhibited in a high K^+ medium (50 mM K^+) (unpublished observation). When K^+ currents were measured using the whole cell patch-clamp method, it was found that charybdotoxin inhibited the carbachol-induced increase in K^+ currents without affecting the carbachol-induced increase in $[Ca^{2+}]_i$ and Cl^- currents (Hirono et al., 1998). By revealing that the carbachol-induced release of calcein is inhibited by charybdotoxin, which suppresses the basolateral K^+ efflux, and by depolarization in a high K^+ medium, these results suggest that anion channels are activated by carba-

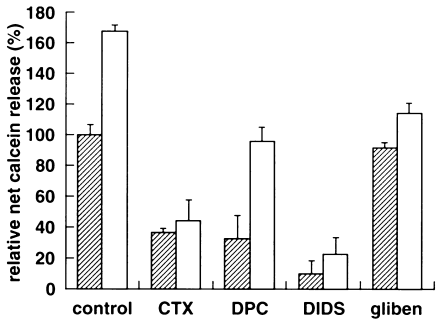

Fig. 4. Effects of ion channel blockers on the agonist-induced release of calcein. 10^{-6} M carbachol (hatched columns) or 10^{-6} M carbachol + 10^{-6} M isoproterenol (open columns) was added to the medium for 10 min in the absence (control) or presence of 10^{-7} M charybdotoxin (CTX), 5×10^{-4} M DPC, 5×10^{-4} M DIDS and 2.5×10^{-4} M glibenclamide (gliben) ($n=3$).

chol and conduct anionic calcein down an electrochemical gradient. The potentiation by isoproterenol of the carbachol-induced release of calcein was also suppressed by charybdotoxin ($P<0.05$; Fig. 4), implying that the isoproterenol-induced potentiation of the release of anionic calcein is also evoked through a coupling with K^+ efflux.

To examine the relationship between the calcein permeation pathways in carbachol-induced release and isoproterenol-potentiated release, we compared the effects of anion channel blockers. The addition of 5×10^{-4} M DPC markedly suppressed the carbachol-induced release of calcein ($P<0.05$; Fig. 4). Although DPC is reported to be a blocker of various Cl^- channels and non-selective cation channels (Poronnik et al., 1991, 1992; Zeng et al., 1997), DPC inhibited the carbachol-induced Cl^- currents, but not the carbachol-induced increase in K^+ currents (Hirono et al., 1998). Nevertheless, DPC had little effect on the potentiation by isoproterenol of the carbachol-induced release of calcein. DIDS, another Cl^- channel blocker (Ishikawa, 1996; Sugita et al., 1998), inhibited the carbachol-induced release of calcein as well as the isoproterenol-induced potentiation ($P<0.05$; Fig. 4). Glibenclamide, known as a blocker of the cystic fibrosis transmembrane conductance regulator (CFTR) and the outwardly rectifying intermediate conductance Cl^- channel (Rabe et al., 1995), did not affect the carbachol-induced release of calcein. However, glibenclamide moderately inhibited the potentiation by isoproterenol of the carbachol-induced release of calcein ($P<0.05$; Fig. 4). Thus, the results of Ca^{2+}-dependence and pharmacology suggest that the calcein release potentiated by cAMP is evoked via a pathway distinct from that for carbachol-induced release.

4. Discussion

Our data indicate that an increase in $[cAMP]_i$ potentiated the carbachol-induced release of calcein. Elevation of $[Ca^{2+}]_i$ is the primary regulator of the carbachol-induced release of calcein (Sugita et al., 1995). Ca^{2+} signaling is governed by the action of Ca^{2+} channels and Ca^{2+} pumps (Lee et al., 1997a,b). Plasma membrane and smooth endoplasmic reticulum Ca^{2+} pumps remove Ca^{2+} from the cytosol, whereas inositol 1,4,5-trisphosphate receptors, ryanodine receptors and capacitative Ca^{2+} entry channels release Ca^{2+} into the cytosol (Chauthaiwale et al., 1996; DiJulio et al., 1997; Lee et al., 1997a; Zhang et al., 1997, 1999; Putney and McKay, 1999). Thus, the coordinated activation and inactivation of these transporters yield $[Ca^{2+}]_i$ signals (Berridge, 1993). It is reported that cAMP can modulate $[Ca^{2+}]_i$ signals by either stimulating or inhibiting Ca^{2+} channels and Ca^{2+}-ATPases (Toyofuku et al., 1993; Dean et al., 1997; Chatton et al., 1998; Tertyshnikova and Fein, 1998; Wojcikiewicz and Luo, 1998). In our study, isoproterenol had little effect on the sustained increase in $[Ca^{2+}]_i$ induced by 10^{-6} M carbachol, but enhanced the calcein release induced by carbachol. Our results suggest that the cAMP-dependent potentiation of calcein release requires a sustained increase in $[Ca^{2+}]_i$, or is regulated by Ca^{2+} entry across the plasma membrane, in contrast with the carbachol-induced release which is triggered by a transient increase in $[Ca^{2+}]_i$ via Ca^{2+} release from intracellular stores.

MRP, an ABC transporter protein, can extrude calcein from the cell against a concentration gradient (Zaman et al., 1994; Keppler et al., 1997; Essodaigui et al., 1998). However, it is likely that both carbachol-induced release and isoproterenol-potentiated release are mediated by channels which conduct anionic calcein down an electrochemical gradient since calcein release was inhibited by charybdotoxin, which suppresses basolateral K^+ efflux, and by depolarization in a high K^+ medium. In addition, the carbachol-induced release of calcein was inhibited by DPC and DIDS, whereas the potentiation by isoproterenol of the carbachol-induced release of calcein was inhibited by DIDS and glibenclamide. Thus, our data indicate that the calcein permeation pathways mediating carbachol-induced release and isoproterenol-potentiated release have distinct sensitivities to anion channel blockers. If the conduction pathways for Cl^- and calcein are distinct, and anion channel blockers inhibit only the Cl^- permeation pathway, the agonist-induced release of calcein might be enhanced by hyperpolarization due to inhibition of the Cl^- permeation pathway, which increases the driving force for anionic calcein. Therefore, a simple explanation for the effects of anion channel blockers is that the blockers directly inhibit the calcein permeation pathways mediating carbachol-induced release and isoproterenol-potentiated release. We cannot exclude the possibility, however, that anion channel blockers inhibit the anion permeation pathway which is closely linked with calcein release, exhibiting an indirect inhibition of calcein release.

The molecular elucidation of the cAMP effects on the carbachol-induced release of calcein and of the functional consequences is complicated by the fact that cAMP potentiated both K^+ and anion conductances activated by an

increase in $[Ca^{2+}]_i$, and the molecular components mediating K^+ and anion transport have not yet been determined. An increase in $[cAMP]_i$ did not induce CFTR-like currents in acinar cells (Hassoni and Gray, 1994; Hirono et al., 1998), which is consistent with the observation that CFTR was not detected by immunofluorescent labelling in salivary acinar cells (He et al., 1997). Therefore, CFTR is not involved in either calcein release or its regulation. H-89 and KT5720, cAMP-dependent protein kinase inhibitors, had little effect on the cAMP-induced potentiation of the carbachol-induced release of calcein, suggesting that protein kinase A-dependent phosphorylation is not involved in the potentiation. How does cAMP potentiate the Ca^{2+}-activated release of calcein? Potentiation by cAMP of the Ca^{2+}-activated K^+ conductance would hyperpolarize the membrane potential (Ishikawa, 1997) and increase the driving force for the release of cytoplasmic anionic calcein. However, most importantly for the present study, our results suggest that the cAMP-dependent potentiation of calcein release is associated with the activation of a pathway distinct from the carbachol-activated one. That the pharmacological properties, time course and $[Ca^{2+}]_i$ dependence vary between carbachol-induced release and isoproterenol-potentiated release argues against the idea that the isoproterenol-potentiated release is mainly attributable to cAMP-dependent modulation of the Ca^{2+}-activated calcein conduction pathway caused by increasing the open probability of the channels or by suppressing channel inactivation.

Further studies will be required to clarify the nature of the calcein permeation pathway and the way in which the cAMP-dependent potentiation of calcein release is regulated by a sustained increase in $[Ca^{2+}]_i$, or by Ca^{2+} entry across the plasma membrane. Since calcein does not have sufficient solubility to be measured as anionic calcein currents by patch-clamping, we have not directly proven that calcein is transported through anion channels. However, our observations indirectly lend support to the idea that anion channels conduct calcein down an electrochemical gradient. When calcein was loaded into intact submandibular glands vascularly perfused, the application of carbachol rapidly evoked the transient release of calcein into the saliva, while carbachol-induced fluid secretion was sustained in two phases. In contrast, the simultaneous addition of carbachol and isoproterenol, following carbachol application, induced the sustained release of calcein into the saliva (unpublished observation). The calcein conduction pathway activated by increases in $[Ca^{2+}]_i$ and $[cAMP]_i$ may be present at the apical membrane. Since increases in $[Ca^{2+}]_i$ and $[cAMP]_i$ induce the sustained release of calcein, the calcein conduction pathway may possibly underlie the release or uptake of structurally related organic anions. MRP mediates the ATP-dependent export of organic anions, in particular glutathione S-conjugates and oxidized glutathione (Leier et al., 1996; Keppler et al., 1997). Transport of glutathione and oxidized glutathione by CFTR, a protein kinase A-regulated Cl^- channel, suggests a functional similarity between CFTR and MRPs (Linsdell and Hanrahan, 1998). The anionic tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) is an extracellular or intracellular antioxidant (Cantin et al., 1987; Aslund and Beckwith, 1999). Glutathione is important for protection against oxidative stress, which generally causes the formation of unwanted disulfide bonds that disrupt protein folding and activity (Aslund and Beckwith, 1999). Glutathione and oxidized glutathione may be transported via the passive calcein conduction pathway activated by increases in $[Ca^{2+}]_i$ and $[cAMP]_i$. Alternatively, the passive calcein permeation pathway, which is sustainedly activated by increases in $[Ca^{2+}]_i$ and $[cAMP]_i$, may be useful to improve chemotherapeutic drug uptake by epithelial tumor cells since calcium and cAMP are ubiquitous intracellular second messengers involved in transduction of extracellular signals. Identification of the molecular basis of the calcein conduction pathway is now of critical relevance for understanding epithelial fluid transport and cellular detoxification.

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