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SECOND MESSENGERS MEDIATING ACTIVATION OF CHLORIDE CURRENT BY INTRACELLULAR GTPγS IN BOVINE CHROMAFFIN CELLS

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SUMMARY

1. Intracellular mechanisms and second messengers involved in chloride current activation by intracellular GTPγS (guanosine 5′-*O*-(3-thiotriphosphate)) in bovine chromaffin cells were studied using the whole-cell patch-clamp technique combined with measurements of intracellular calcium $[Ca^{2+}]_i$.

2. No correlation between the time of current activation and the appearance of $[Ca^{2+}]_i$ transients was observed; intracellular introduction of sufficient EGTA (10 mM) to suppress the $[Ca^{2+}]_i$ transients did not affect the current activation by GTPγS.

3. The cyclic nucleotides, cyclic AMP or cyclic GMP, did not activate the current when introduced intracellularly (50–250 μM). The ability of GTPγS to activate the current decreased when cyclic GMP (250 μM), together with MgATP (2 mM), was added to the perfusate.

4. Neomycin (0·5–1 mM), a presumed inhibitor of phospholipase C effectively prevented the current activation by GTPγS but it did not prevent $[Ca^{2+}]_i$ transients.

5. Modulation of protein kinase C activity using specific inhibitors (H-7, 300 μM; polymyxin B, 400 U/ml) or activators (phorbol ester PMA, 100 nM, 20–90 min at 37 °C) did not affect the current activation by GTPγS nor did it cause current activation in the absence of GTPγS.

6. Activation of the current by GTPγS could be prevented by incubating the cells for 10–15 min with 2·5 μM *p*-bromophenacyl bromide (*p*-BPB), an inhibitor of phospholipase A_2 activity. Exogenous arachidonic acid (5–10 μM), applied extracellularly or intracellularly, neither activated the current itself nor did it interfere with its activation by GTPγS.

7. Activation of the current by GTPγS could also be prevented by incubating the cells with 1 μM-nordihydroguaiaretic acid (NDGA), an inhibitor of lipoxygenase, but not with indomethacin (2 μM), an inhibitor of cyclo-oxygenase pathway of arachidonic acid metabolism.

8. It is suggested that chloride current activation by GTPγS in bovine chromaffin

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cells involves G protein-mediated stimulation of phospholipase A_2 activity and subsequent formation of lipoxygenase product(s) of arachidonic acid metabolism.

INTRODUCTION

In the preceding paper (Doroshenko, Penner & Neher, 1991), a transient current was described which originates from Cl^--selective conductance. It is activated in the membrane of bovine chromaffin cells following perfusion with GTPγS (guanosine 5′-*O*-(3-thiotriphosphate)-containing intracellular solutions. The involvement of a G protein was postulated which, however, does not act directly on the Cl^--selective channels in the cellular membrane but instead via some second messenger, probably generated by G protein-activated enzymes.

G proteins are involved in receptor-driven modulation of cellular metabolism by every known second messenger: cyclic AMP via activation or inhibition of adenylate cyclase, cyclic GMP via activation of the cyclic GMP phosphodiesterase (reviewed by Stryer & Bourne, 1986; Gilman, 1987), Ca^{2+} ions and diacylglycerol via stimulation of phospholipase C (Cockcroft & Gomperts, 1985; Merritt, Taylor, Rubin & Putney, 1986; Cockcroft & Stutchfield, 1988), and also arachidonic acid and its metabolites via activation of phospholipase A_2 (reviewed by Fain, Wallace & Wojcikiewicz, 1988). Each of these substances has been shown to be involved in ion channel activation. For example, there are cyclic AMP-activated sodium channels in snail neuron membranes (Aldenhoff, Hofmeier, Lux & Swandulla, 1983), cyclic GMP-activated cationic conductance in retinal rod outer segment membranes (Fesenko, Kolesnikov & Lyubarsky, 1985), Ca^{2+}-activated potassium channels in membranes of bovine chromaffin cells (Marty, 1981), and chloride-selective channels in *Xenopus* oocytes (Takahashi, Neher & Sakmann, 1987). Recently, evidence has been provided implicating arachidonic acid and its metabolites in the activation of potassium channels in cardiac cells (Belardetti & Siegelbaum, 1988; Kim & Clapham, 1989; Kurachi, Ito, Sugimoto, Shimuzu, Miki & Ui, 1989).

The GTPγS-activated chloride channels in bovine chromaffin cells resemble, in some properties, the delayed chloride current channels in rat mast cell membrane (Matthews, Neher & Penner, 1989) and the Cl^--selective channels involved in volume regulation by lymphocytes (see, for example, Cahalan & Lewis, 1988). The former have been shown to be activated by intracellularly applied cyclic AMP (Matthews *et al.* 1989). Evidence for dependence of these Cl^--selective channels, which have a very low single-channel conductance, on $[Ca^{2+}]_i$ ions either has not been demonstrated (Hazama & Okada, 1988) or has been considered indirect (Matthews *et al.* 1989). There is, however, evidence for the involvement of arachidonic acid metabolites, specifically leukotriene D_4, in activating chloride channels responsible for volume regulation in Ehrlich ascites tumour cells (Lambert, 1987).

The aim of the present work was to investigate the effects of experimentally induced perturbances in metabolism of some presumed second messengers on GTPγS-mediated activation of chloride channels in bovine chromaffin cells.

METHODS

Experiments were performed on bovine chromaffin cells in primary culture (Fenwick, Marty & Neher, 1982) using whole-cell patch-clamp and $[Ca^{2+}]_i$ measurement techniques (see companion

paper, Doroshenko *et al.* 1991; also Neher, 1989). The extracellular solution (standard saline) used contained (in mM): 140 NaCl, 2·8 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES-NaOH at pH 7·2. The intracellular solution (standard internal solution) contained (in mM): 145 potassium glutamate, 8 NaCl, 1 $MgCl_2$, 0·5 MgATP, 10 HEPES-KOH at pH 7·2, 0·1 Fura-2. The Tris-based internal solution contained (mM): 140 Tris-HCl, 1 $MgCl_2$, 10 HEPES-NaOH at pH 7·2, 0·1 Fura-2. Chemicals used were: nordihydroguaiaretic acid (NDGA, 4,4′-(2,3-dimethyl-1,4-butanediyl)-bis(1,2-benzenediol)), 4-bromophenacyl bromide (p-BPB, 2,4-dibromoacetophenone), indomethacin (1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid), the phorbol esters phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate (PDBu), neomycin sulphate, polymyxin B sulphate, ethylene glycol-bis (β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), and arachidonic acid, all from Sigma; H-7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride) from Seikagaku Kogyo, Tokyo, Japan; Fura-2, pentapotassium salt, from Molecular Probes, Eugene, OR, USA; GTPγS (guanosine-5′-*O*-(3-thiotriphosphate), tetralithium salt, from Boehringer, Mannheim. H-7 and polymyxin B were dissolved in H_2O at 10 mM and 40000 U/ml concentrations, respectively. Arachidonic acid was dissolved (10 mM) in ethanol under argon protection. NDGA, indomethacin and *p*-BPB were dissolved (10 mM) in dimethyl sulphoxide (DMSO) and sonicated for about 3 min. Before use, the drugs were further diluted to their final concentrations in standard saline or Tris-based intracellular solutions; those dissolved in DMSO were diluted in two steps: first, to 50 μM then to 1–2 μM, each time in standard saline followed by sonication. Final concentration of DMSO in the experimental solutions was less than 0·02%. Non-specific effects of DMSO on the GTPγS-activated current were ruled out by control experiments with DMSO in the bath solution.

RESULTS

Calcium dependence of the GTPγS-activated current

Cytoplasmic Ca^{2+} ions serve as second messengers in many cellular processes. There are numerous examples of different Ca^{2+}-activated ionic channels, among them Cl^--selective channels (Takahashi *et al.* 1987). As GTPγS evokes $[Ca^{2+}]_i$ transients in parallel with the chloride current, current activation might be secondary to an increase in $[Ca^{2+}]_i$. The data do not support this suggestion, however. First, in comparable experimental conditions either current (Fig. 1*A*) or $[Ca^{2+}]_i$ transients (Fig. 1*D*) could be observed in the absence of the corresponding counterpart. Second, the time of the current activation and the appearance of $[Ca^{2+}]_i$ transients was not correlated. There were cells in which $[Ca^{2+}]_i$ oscillations preceded activation of the current (Fig. 1*B*) and, vice versa, in which the current started to increase before the $[Ca^{2+}]_i$ transients appeared (Fig. 1*C*). Third, large changes in $[Ca^{2+}]_i$ during the stimulated transients did not affect the current unless K^+ ions were present to carry Ca^{2+}-activated K^+ currents. Fourth, adding 10 mM-EGTA to the intracellular solution, enough to completely eliminate the $[Ca^{2+}]_i$ transients, had no obvious effect on the probability of the current activation (systematic investigations of EGTA effects on the time course or the peak amplitude of the current were not performed). Possible stimulating effects of prolonged action of high levels of $[Ca^{2+}]_i$ (similar to that described for the chloride current in mast cells, Matthews *et al.* 1989) cannot be excluded because such experiments are inconclusive due to morphological changes, that take place under these conditions.

Role of nucleotides

The cyclic nucleotides, cyclic AMP and cyclic GMP, are often involved in mediating activation of ion conductances. Whereas cyclic AMP has been shown to activate the delayed chloride current in mast cell membranes (Matthews *et al.* 1989), this is apparently not the case with the GTPγS-activated current in chromaffin cells.

Addition of cyclic AMP (50–250 μM) to the intracellular solutions did not cause the current activation: only one out of sixteen cells produced a short-duration inward current of small amplitude under these conditions. Similar results were obtained with cyclic GMP (50–250 μM): slowly developing inward current with small variance,

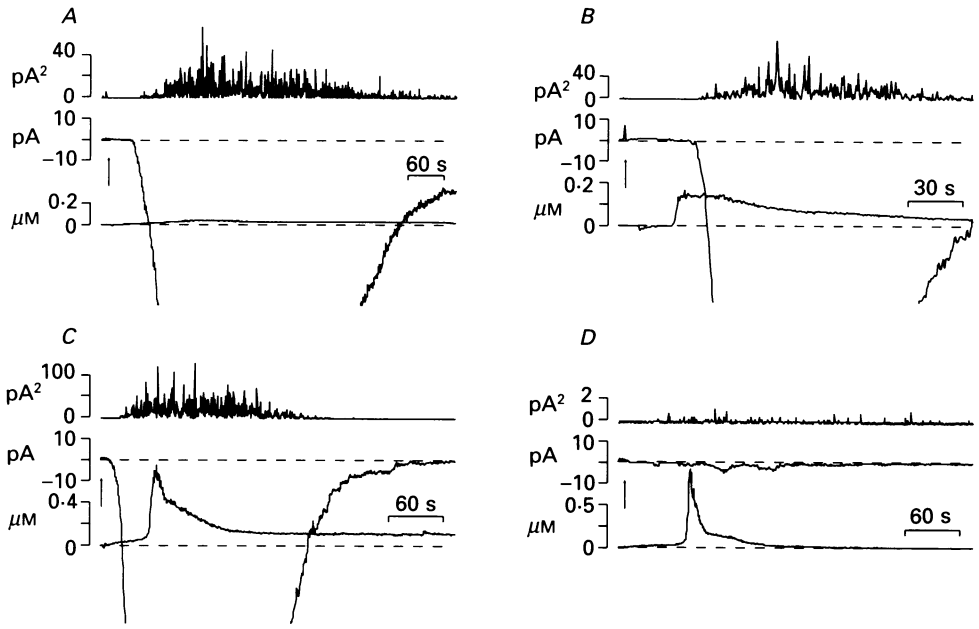

Fig. 1. Demonstration of the absence of correlation between the GTPγS-induced chloride current and intracellular Ca^{2+} transients. Panels *A–D* show examples of different temporal relationships between the current activation and $[Ca^{2+}]_i$ transients during perfusion of cells with GTPγS-containing Tris-based internal solutions (50 μM-GTPγS + 300 μM-GTP in *A*, 200 μM-GTPγS in *B*, 5 μM-GTPγS in *C* and 5 μM-GTPγS + 300 μM-GTP in *D*). The extracellular bath solution was standard saline here and in subsequent figures except where indicated. All cells shown were from the same preparation. Holding potential was −60 mV in all experiments. The traces (from top to bottom) show the current variance, current and $[Ca^{2+}]_i$. Dashed lines show the corresponding zero levels.

similar to that for GTPγS-activated current, was observed in only two of twenty-eight cells. Taking into account the possibility of the spontaneous activation of the current, one can conclude that neither of the cyclic nucleotides directly induces the current under study.

Furthermore, cyclic nucleotides could partially prevent activation of the current by GTPγS. Figure 2 demonstrates the effects of exogenous cyclic nucleotides on current activation by GTPγS. Each panel presents data from a single culture dish. Each bar represents a single-cell experiment in the sequence actually performed. A bar directed upward indicates current activation (the current amplitude was not taken into account), a bar directed downward indicates no current activation. In fact, adding cyclic AMP seemed to make little difference; $[Ca^{2+}]_i$ transients, however, were significantly larger with cyclic AMP. On the other hand, addition of cyclic GMP

produced a clear depressing effect (Fig. 2*A*): only two out of ten cells produced the current, while five out of seven cells from the same cover-glass perfused with cyclic AMP-containing solution produced the current. This depressing effect was corroborated in another experiment (Fig. 2*B*) in which cells were perfused with either

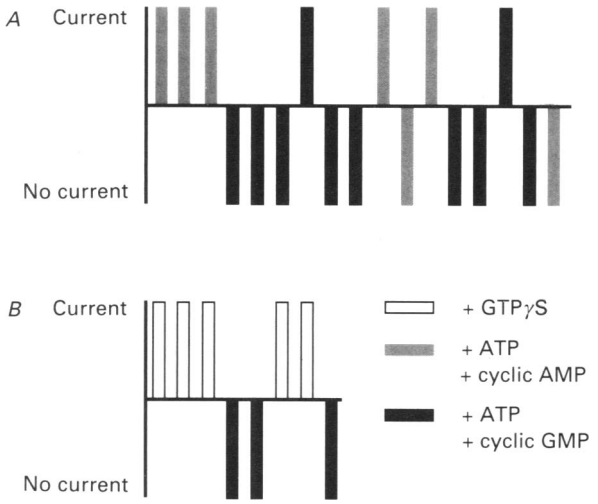

Fig. 2. Influence of nucleotides on activation of the current by GTPγS. Panels *A* and *B* present two different experiments each carried out on cells from the same cover-glass. Each bar represents a single-cell experiment: bars directed upward indicate that the chloride current was generated in that experiment; bars directed downward indicate that the current was not observed (the current amplitude was not taken into account). Cells were perfused with Tris-based internal solution containing 200 μM-GTPγS with different additions (reflected in the different shading of the bars as indicated).

(Tris + GTPγS) solution or (Tris + GTPγS + MgATP + cyclic GMP) solution: all cells ($n = 5$) perfused with the first solution produced the current while those ($n = 3$) perfused with the second solution did not. The presence of MgATP (2 mM) in the intracellular perfusate affected neither amplitude nor time to peak of the GTPγS-activated current: in a paired-cell experiment on six cells these were 336 ± 175 pA and 85 ± 30 s, respectively, in the presence of 2 mM-MgATP and 447 ± 315 pA and 62 ± 2 s in its absence. In some preparations, however, addition of MgATP to the intracellular perfusate seemed to slightly decrease the efficacy of the current activation by GTPγS.

Possible role for protein kinase C

A probable target of the activated G protein is phospholipase C whose cleavage products mediate the simultaneous processes of Ca^{2+} release from intracellular stores (Berridge, 1984) and protein kinase C activation (Nishizuka, 1986).

It is unlikely that any protein kinase is involved in the GTPγS-mediated current activation since the current was still present when MgATP was eliminated from the intracellular perfusate. In addition, drugs which inhibit protein kinase C, H-7

(300 μM) and polymyxin B (400 U/ml), did not prevent current activation by intracellular GTPγS whether applied outside (bath application) or inside the cell. Pre-treatment of cells with the phorbol ester PMA also failed to prevent current activation. Cells were incubated with 100 nM-PMA at 37 °C for 20, 45 or 90 min. Most

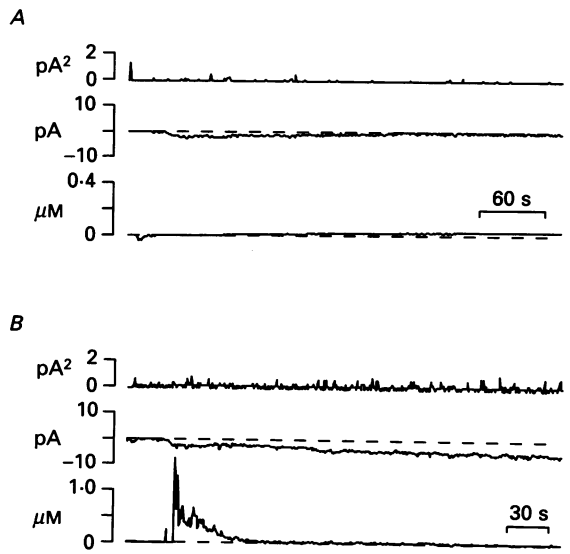

Fig. 3. Inhibition of the GTPγS-activated current by neomycin. *A*, lack of effects of the drug itself in the absence of GTPγS-stimulation. The cell was perfused with Tris-based internal solution plus 1 mM-neomycin. *B*, typical results when the cell was perfused with Tris-based internal solution + 200 μM-GTPγS plus 1 mM-neomycin. Traces in *A* and *B* show (from top to bottom) current variance, membrane current, $[Ca^{2+}]_i$.

of the pre-treated cells retained their normal appearance, while some of them developed round 'vacuoles' in their cytoplasm and looked pale. Of the treated cells, seventeen out of nineteen produced both the current and $[Ca^{2+}]_i$ transients during intracellular perfusion with GTPγS. Applications of PMA (10 μM for 300 s from a puffer pipette) or of 100 nM-PDBu, a membrane-permeable analogue of PMA, during intracellular perfusion of cells with the control solutions (i.e. without GTPγS) did not cause the current activation either.

Inhibition of the current by neomycin

The above results indicate that none of the signals generated by phospholipase C activity is involved in eliciting the GTPγS-activated current. It is therefore expected that inhibition of phospholipase C should be without effect. To our surprise, however, neomycin, an inhibitor of the enzyme appeared to be very effective in blocking the GTPγS-activated current. It did not produce any effect on control cells (Fig. 3*A*) but prevented activation of the current by GTPγS. No cell out of several dozens of cells from different preparations perfused with Tris-based internal solution, containing GTPγS plus 0·5–1 mM-neomycin produced the current (Fig. 3*B*). At the same time,

the GTPγS-induced $[Ca^{2+}]_i$ transients were affected little. This contradicts the generally accepted mechanism of action of the drug, according to which neomycin, above all, inhibits formation of inositol 1,4,5-trisphosphate (IP_3) by avidly binding to polyphosphoinositides, thus making them unavailable to phospholipase C.

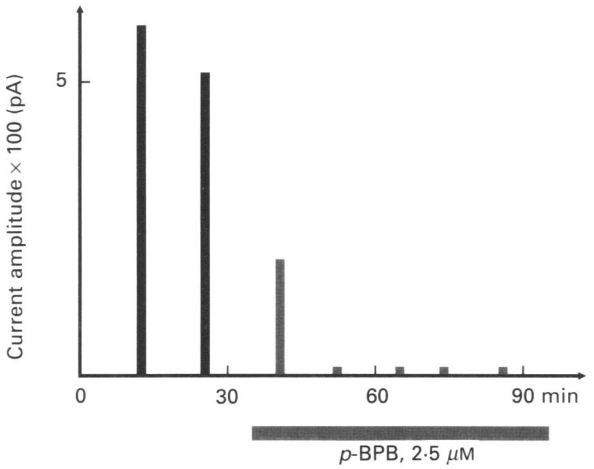

Fig. 4. Inhibition of the GTPγS-activated current by *p*-bromophenacyl bromide. Results of a series of single-cell experiments carried out on cells from the same cover-glass. Each bar represents a single-cell experiment: the bar's position on the *X*-axis shows the time of the experiment, the bar height shows the amplitude of the current. Cells were perfused with Tris-based internal solution + GTPγS (100 μM). The horizontal bar denotes addition of 2·5 μM-*p*-BPB. The last four bars are not to scale and indicate the experiments where current development was not seen. Current was not restored 60 min after wash-out (not shown).

However, the possibility exists that neomycin cannot act fast enough, while it is diffusing into the cell together with GTPγS.

Arachidonic acid involvement

To assess the effect of phospholipase A_2 on current activation, we used drugs which act on arachidonic acid pathways: nordihydroguaiaretic acid (NDGA), *p*-bromophenacyl bromide (p-BPB) and indomethacin (Rainsford, 1988; Okada, Yamagishi & Sugiyama, 1989). *p*-Bromophenacyl bromide is known to inhibit phospholipase A_2 activity (Hofmann, Prescott & Majerus, 1982). Incubation of bovine chromaffin cells ($n = 23$, four preparations) in solutions containing 1–10 μM-*p*-BPB inhibited the GTPγS-stimulated current (see Fig. 4). These experiments were performed in the following manner. First cells were bathed in standard saline. Several cells from a given cover-glass were tested for their ability to generate the current in response to intracellular GTPγS (usually 100 μM). Following addition of the drug to the bath, new cells from the same cover-glass were tested as before. Following drug wash-out other cells from the same cover-glass were assayed.

With 2·5–5 μM-*p*-BPB in the bath, the GTPγS-activated current was fully depressed after 10–15 min of incubation (Fig. 4). The ability of cells to generate

$[Ca^{2+}]_i$ transients in response to the intracellular introduction of GTPγS persisted longer (approximately 1 h) but was also lost later. The effect of *p*-BPB was apparently irreversible; 1 h after wash-out of *p*-BPB the cells failed to recover their ability to generate either the current or $[Ca^{2+}]_i$ transients in response to perfusion

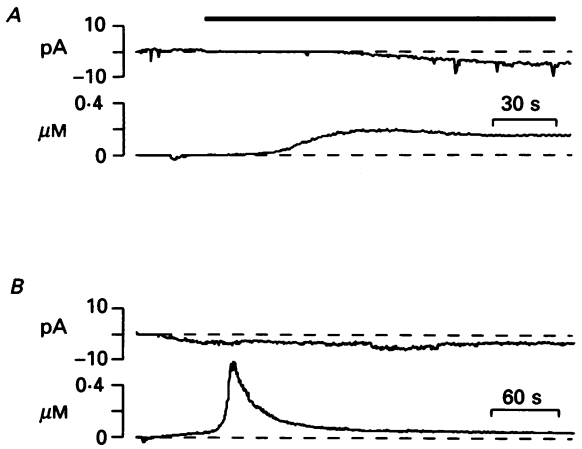

Fig. 5. Effects of exogenous arachidonic acid on perfused chromaffin cells. Upper trace is current; lower trace is calcium concentration. *A*, effects of arachidonic acid (10 μM) application on a cell perfused with Tris-based internal solution. The time of application is indicated with the horizontal bar above the current trace. *B*, effects of intracellular introduction of arachidonic acid. The cell was perfused with Tris-based internal solution + 10 μM-arachidonic acid.

with GTPγS. It is worth mentioning that the cells had normal appearance in the presence of *p*-BPB, the giga-seals were formed easily, the break-in and the Fura loading proceeded as in the control case. The above results suggest that arachidonic acid (AA) or its metabolites might be involved in inducing current expression, but exogenous AA applied from a puffer pipette (10 μM, four cells) did not induce the current in cells perfused with the control Tris-based internal solution (Fig. 5*A*). Shallow $[Ca^{2+}]_i$ transients of small amplitude were observed. When added to the intracellular perfusate (10 μM, seven cells), AA did not activate the current. However, $[Ca^{2+}]_i$ increased during the perfusion (occasionally as a single peak up to 0·6 μM at $t = 70$–100 s; Fig. 5*B*). No extra effect of 5 μM-AA was observed when used in the intracellular perfusate together with GTPγS.

Cyclo-oxygenase or lipoxygenase pathway

To further differentiate between possible mechanisms of activation we examined effects of two drugs reported to selectively affect the two main pathways of arachidonic acid metabolism: NDGA which inhibits activity of lipoxygenase and indomethacin which preferably inhibits activity of cyclo-oxygenase (Rainsford, 1988). We found that incubation of cells ($n = 32$) with NDGA at concentrations as low as 0·5–2·5 μM blocked current activation by GTPγS. In a typical experiment using 1 μM-NDGA in the bath, the ability of cells to produce the GTPγS-activated current was significantly impaired within 10–30 min (Fig. 6). Figure 7*A* shows the

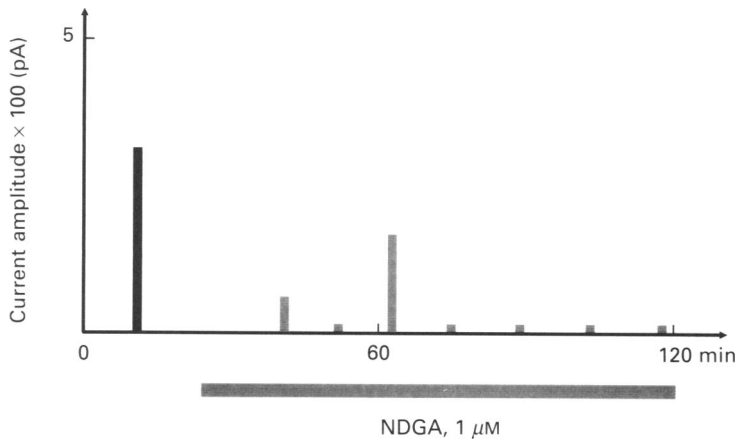

Fig. 6. Time course of inhibition of the GTPγS-activated current by NDGA. Cells were bathed in standard saline to which 1 μM-NDGA was added during the time indicated (horizontal bar) and perfused with Tris-based internal solution + GTPγS (100 μM). The experimental procedure was similar to that described in the legend of Fig. 4. The third and fifth to eighth bars are not to scale; they just mark the times of experiments in which the current was not observed.

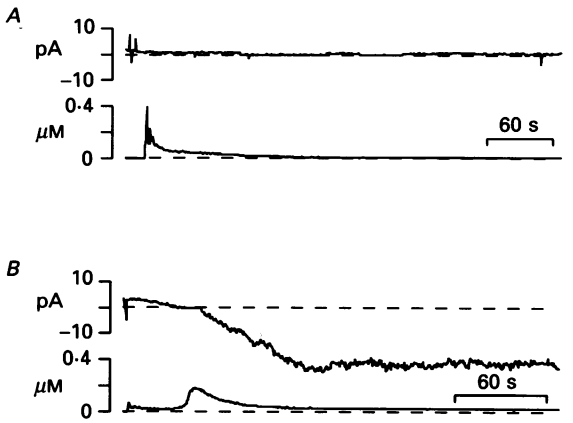

Fig. 7. Inhibitory effects of NDGA on the GTPγS-activated current. *A*, typical recordings from the cell after long-lasting (50 min) incubation with 1 μM-NDGA added to standard saline during perfusion with Tris-based internal solution + GTPγS (100 μM). *B*, results of an experiment with extracellular application of NDGA (5 μM) on a cell perfused with Tris-based internal solution + GTPγS (100 μM). The application started 1 min before the break-in; during the recording time it is indicated by a horizontal bar above the traces. Traces here are ordered as in Fig. 5.

current and $[Ca^{2+}]_i$ traces recorded in this experiment from a cell after 50 min of incubation with NDGA. The effects of NDGA on the GTPγS-activated current were largely irreversible.

Applications of NDGA (5 μM) from a puffer pipette during current development had no effect (not shown). When the application was started 1 min prior to break-in, however, current activation by GTPγS was largely retarded (Fig. 7*B*).

On the other hand, experiments where cells ($n = 14$, 2 preparations) were incubated with indomethacin, an inhibitor of cyclo-oxygenase, revealed significant differences in effect when compared to NDGA. When cells were perfused with a solution containing 100 μM-GTPγS the amplitude of the GTPγS-activated current became

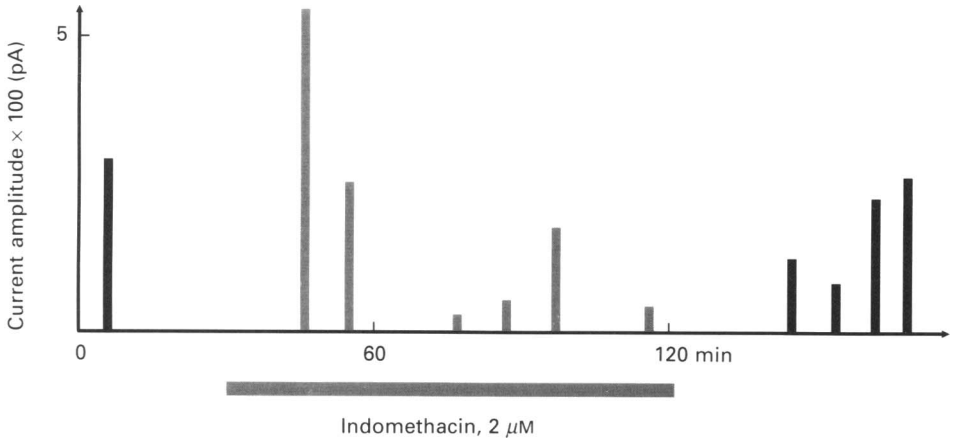

Fig. 8. Effects of indomethacin on the GTPγS-activated current. Cells were perfused with Tris-based internal solution + GTPγS (100 μM) and bathed in standard saline. Horizontal bar under the X-axis shows time of addition of 2 μM-indomethacin to the bath. The experimental procedure is similar to that of Fig. 4.

progressively smaller following incubation with saline containing 2 μM-indomethacin (Fig. 8). However the current still was not fully inhibited after approximately 1·5 h. Furthermore, the drug effect was reversible; when indomethacin was removed from the bath, the current amplitude was partially restored. Intracellular [Ca^{2+}] transients remained largely unchanged when indomethacin was present. These results indicate that cyclo-oxygenase is probably not involved in the current activation contrary to the case of lipoxygenase.

DISCUSSION

The experiments presented here were designed to elucidate signalling mechanism which induced the GTPγS-activated chloride current in bovine chromaffin cells. Ca^{2+} ions, cyclic AMP, cyclic GMP, activators and inhibitors of protein kinase C, as well as inhibitors of arachidonic acid metabolism were tested for their ability to activate the current or to affect its activation by GTPγS.

Ion channels which are activated by intracellular Ca^{2+} ions, including Cl^--selective channels, have been found in various cell types (Marty, 1989). In bovine chromaffin cells large-conductance Ca^{2+}-activated K^+ channels have been described (Marty, 1981). In our experiments, activation of the chloride current by GTPγS in chromaffin cells did not appear to be related to $[Ca^{2+}]_i$ increase since no correlation was observed between the two processes. Also, lowering $[Ca^{2+}]_i$ by means of EGTA affected neither the current's time course nor its amplitude. In this respect, the GTPγS-activated channels in bovine chromaffin cells are similar to Cl^--selective channels involved in regulatory volume changes in human epithelial cells (Hazama & Okada, 1988).

In contrast to chloride channels in mast cells, where cyclic AMP is very effective in the current activation (Matthews *et al.* 1989), the stimulating action of intracellular GTPγS on the chloride channels in chromaffin cells could not be mimicked by exogenous cyclic nucleotides. Neither cyclic AMP nor cyclic GMP was able to activate the current. Furthermore, adding cyclic AMP or cyclic GMP to the intracellular perfusate decreased the probability of current activation by GTPγS. This depressing effect, particularly evident with cyclic GMP, may be ascribable to the action of protein kinases, since, in some preparations, MgATP by itself decreased the probability of current activation by GTPγS. GTP-binding proteins mediate hormonal stimulation of enzymes involved in phospholipid turnover, phospholipase C (Merritt *et al.* 1986) and phospholipase A_2 (Burch, Luini & Axelrod, 1986; Burch & Axelrod, 1987; Jelsema, 1987). Cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C yields IP_3 which stimulates Ca^{2+} release from intracellular stores (Berridge, 1984) and 1,2-diacylglycerol, which activates protein kinase C (Nishizuka, 1986). Appearance of GTPγS-induced $[Ca^{2+}]_i$ oscillations may indicate phospholipase C activation, as IP_3 has been shown to mediate Ca^{2+} release from intracellular stores in bovine chromaffin cells (Stoehr, Smolen, Holz & Agranoff, 1986). Modulation of protein kinase C activity using inhibitors (H-7 or polymyxin B) or activators (phorbol esters) did not affect the current. Contrary to expectations, neomycin (0·5–1 mM) did not block GTPγS-induced $[Ca^{2+}]_i$ transients in bovine chromaffin cells, but appeared to be effective in inhibiting the GTPγS-activated current. It is believed that neomycin selectively binds to PIP_2 (Reid & Gajjar, 1987) making it unavailable as a substrate for phospholipase C. Neomycin has been shown to inhibit a variety of IP_3-dependent cellular processes (Cockcroft & Gomperts, 1985; Slivka & Insel, 1988; Diamant, Lev-Ari, Uzielli & Atlas, 1988). The experimental observations contradict expectations based on the suggested activation of phospholipase C, and thus do not support such a hypothesis. However, it may be argued that when neomycin is applied simultaneously with GTPγS in the whole-cell experiment, it cannot act fast enough to prevent phospholipase C activation. Positive evidence was obtained, however, for the involvement of phospholipase A_2 in the current activation by GTPγS, since *p*-BPB inhibited GTPγS-mediated current activation. This drug has been shown to interfere with phospholipase A_2 activity in different cells (for example: human platelets, Hofmann *et al.* 1982; rat hippocampal neurons, Okada *et al.* 1989). Recent investigations have shown that arachidonic acid and its metabolites which are products of phospholipase A_2 activity can modulate ion channel activity (Belardetti & Siegelbaum, 1988; Kim & Clapham, 1989; Kurachi *et al.* 1989). Arachidonic acid has been reported to mobilize intracellular Ca^{2+} ions in pancreatic islets with a molar potency similar to that of IP_3 (Wolf, Turk, Sherman & McDaniel, 1986). Exogenous arachidonic acid whether applied extracellularly or intracellularly, did not activate the current by itself nor did it affect activation by GTPγS, although it did increase $[Ca^{2+}]_i$. As it has been shown that exogenous AA is not readily metabolized by cellular enzymes (Anggard, 1988), it may be that it is not AA but its metabolites which activate the current. Inhibition of current activation by NDGA, an inhibitor of the lipoxygenase pathway of arachidonic acid metabolism, suggests a role for leukotrienes. Indomethacin, a presumed inhibitor of cyclo-oxygenase (Brune, Rainsford, Wagner & Peskar, 1981), was far less effective in

suppressing the GTPγS-activated current. This distinction depends largely on selectivity of the drug's action. Indeed, concentrations of NDGA necessary for a 50% inhibition of a lipoxygenase are reported to be orders of magnitude smaller than for a cyclo-oxygenase, while the reverse is true for indomethacin. For example, the effective dose range for inhibition of leukotriene B_4 production in rat leucocytes has been estimated at 100 μM for indomethacin and 0·1–10 μM for NDGA (see Rainsford, 1988).

Thus, it seems reasonable to suggest that the activation of the chloride current in membrane of chromaffin cells by intracellular GTPγS occurs via G protein-mediated stimulation of phospholipase A_2 and the subsequent formation of effectors possibly leukotrienes. Recently, it has been shown that leukotriene D_4 specifically affected the volume regulation in Ehrlich tumour cells by stimulating K^+ and Cl^- volume-regulated conductances (Lambert, 1987). The $[Ca^{2+}]_i$ transients observed in parallel with the current activation might partly originate from arachidonic acid metabolism rather than exclusively from IP_3 production.

The current described here shares several properties with chloride currents in rat mast cells (Matthews *et al.* 1989), and those involved in volume regulation by lymphocytes (Cahalan & Lewis, 1988) and epithelial cells (Hazama & Okada, 1988); these include activation by GTPγS, block by micromolar concentrations of DIDS, small single-channel conductance, similar ion selectivity, and slow time course of development. The similarities suggest that the same kind of channel is active in all three cases. There are, however, important differences. The current in chromaffin cells cannot be activated by cyclic AMP (such as in mast cells), and it is transient in nature. It is presently unclear whether these differences require distinct channel types or merely reflect different mechanisms of channel modulation in different cell types.

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